Ti plasmid and the Future of Genetically Modified Plants

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Abstract

Ti plasmid-mediated plant transformation, utilizing the natural gene transfer machinery of *Agrobacterium* bacteria, has revolutionized our ability to introduce and express desired genes in plants. This highly efficient and versatile technique has facilitated the creation of genetically modified (GM) crops that have improved tolerance to pests, diseases, and herbicides, improved nutritional value, and tolerance to environmental stresses. Despite its undeniable success, concerns regarding potential unintended consequences and ethical implications have led to ongoing public debate.

This abstract delves into the fundamentals of Ti plasmid-mediated transformation, exploring the intricate bacterial-plant interaction and highlighting its advantages over alternative methods. It then examines the diverse applications of this technology in agriculture, medicine, and bioremediation, showcasing the potential benefits of GM plants for global food security, sustainable agriculture, and environmental protection.

However, the abstract acknowledges the ethical and regulatory concerns surrounding GM technology, including potential risks to biodiversity, human health, and the environment. It emphasizes the importance of robust scientific research, transparent communication, and open dialogue to address these concerns and ensure the responsible development and deployment of GM plants.

Keywords: Ti plasmid, *Agrobacterium*, plant transformation, genetically modified crops, agriculture, bioremediation, ethics, future of food.

1. Introduction

Agrobacterium tumefaciens, a pathogen found in soil, attacks plants at their wound sites and induces Crown Gall disease. This is achieved by transferring T-DNA (Transferred DNA) from the bacterium into the plant's genome via a bacterial type IV secretion system (T4SS). Throughout the past few decades, many advancements and innovations in the field of molecular biology and its technologies have been instrumental in discovering many critical bacterial and plant genes associated with tumor formation. With the aid of a better comprehensive understanding of the interactions taking place between A. tumefaciens and the plant host cells, this has

emerged as the most widely utilized tool for plant transformation to date [1].

2. Molecular basis for *Agrobacterium*-Mediated Transformation

The molecular basis for the genetic modification of plant cells through transformation by *Agrobacterium* is the transfer of a tumour-inducing (Ti) region in *Agrobacterium sp*. The T-DNA, also referred to as T-region when present on the Ti plasmid, represents only a small percentage of the plasmid, at an approximate size of 10-30 kbp [2].

There are two necessary components present within the Ti plasmid of *Agrobacterium tumefaciens* that facilitates the plant genetic transformation process. The first of these factors is the T-DNA border sequences. These are highly homologous sequences of approximately 25 bp in length that are situated at the ends of the T-region – one on the right side called the right border sequence and the other the left border sequence present on the left side of the DNA. The second factor responsible is the virulence or *vir* region of the T-plasmid.

2.1 Virulence region

The *vir* region constitutes several operons that serve four major roles.

(a) Recognition of plant compounds and activation of *virA* and *virG*: - When a plant gets wounded, it releases special molecules called phenolic compounds [3]. Certain phenolic compounds are recognized by *Agrobacterium*, and it initiates a special response system. This system is made up of two components – *virA* and *virG*. *virA* gets activated by these compounds and it initiates a sequence of reactions. These reactions ultimately lead to the phosphorylation and activation of *virG* [4]. This activated *virG* then binds to each *vir* gene operon, increasing their production [5].

(b) Processing of T-DNA by *virD1* and *virD2*: - VirD1, a helicase, and VirD2, an endonuclease, jointly known as VirD1/D2, bind to and nick the T-DNA border sequences [6][7]. The VirD2 protein establishes a covalent bond with the 5’ terminus of the processed ss T-strand DNA. It subsequently functions as a chaperone to facilitate the DNA translocation from the bacterial cytoplasm to the plant cell nucleus. This process involves the egress of the VirD2-T-strand complex from the bacterium, followed by its entry into the plant cell and subsequent targeting of the nucleus [8][9].

(c) Secretion of T-DNA and Vir proteins from the bacterium via T4SS operated by *virB* operon and *virD4*: - The *Agrobacterium tumefaciens* VirB operon, encompassing a total of eleven genes, encodes core components that assemble into a Type IV secretion

system or T4SS across the bacterial inner and outer membranes. This system facilitates the translocation of vir proteins from the bacterial cytoplasm into the plant cell cytoplasm, enabling their participation in the transfer of bacterial DNA [10]. To date, five distinct vir proteins have been identified as substrates for this secretion system: both unattached and T-strand-associated virD2, virD5, virE2, virE3, and virF [11][12]. Additionally, VirD4 serves as an essential chaperone and a dedicated adaptor protein, mediating a critical physical link between the VirD2-T-strand complex and the T4SS, thereby ensuring its efficient export from the bacterium [10].

(d) Participation of different *vir* proteins within the host cell: - The VirD2 and VirE2 proteins are suspected to be involved in the nuclear targeting of the processed ss DNA (T-strand) within the plant cell [9][13]. Additionally, VirE2 is postulated to shield the T-strand from nucleolytic degradation within the plant cytoplasm, thereby preserving its integrity [14][15]. Furthermore, VirF may contribute to the removal of associated proteins from the T-strand, potentially facilitating its subsequent T-DNA integration into the plant genome [16][17].

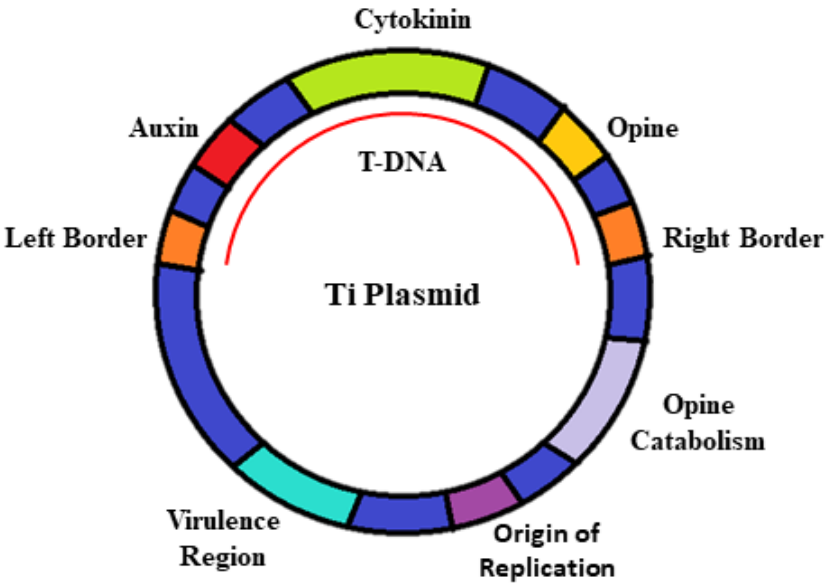

Fig 1. Schematic representation of a typical Ti plasmid

3. T-DNA Transfer into the Host Plant Cell

The VirB/D4 type IV secretion system is employed by *Agrobacterium tumefaciens* to transport the complex of VirD2-T-strand DNA and effector proteins into the cells of the host plant. One such effector, VirE2, is a ss DNA-binding protein or SSB. Once translocated, VirE2 cooperatively binds to the T-strand, resulting in the formation of a particle known as the T-complex, which includes the VirD2-T-strand-VirE2 assembly [18]. VirD2 and VirE2 both play crucial roles in ensuring the T-complex is effectively transported to the plant cell's nucleus.

Crucially, both proteins contain nuclear localization sequences (NLSs), which aid in directing the T-complex to the plant cell nucleus by interacting with specific plant proteins. Significantly, VirD2 and VirE2 engage with importin α family proteins, serving as adaptors that identify NLS motifs and collaborate with the nuclear shuttle protein importin β, facilitating the nuclear import process [19] [20].

Moreover, VirD2, as a phosphoprotein, engages with and undergoes phosphorylation by CAK2Ms, which are cyclin-dependent kinase-activating kinases [21]. CAK2Ms are also responsible for phosphorylating the large sub-units of RNA polymerase II, a step that aids in attracting a TATA-Box binding protein essential for the commencement of transcription. This interaction implies that the phosphorylation of VirD2 may be involved in directing T-complexes to chromatin.

Additionally, VirD2 engages with cyclophilins found in plants. They are proteins involved in protein folding, although the specific function of this interaction remains unclear [22] [23].

Intriguingly, VirE2 exhibits gated channel formation in black lipid membranes, prompting speculation that it may facilitate the entry of the T-complex through the plant's cytoplasmic membrane via channel formation. [24].

Within the host cell nucleus, the VirD2 nuclear localization sequence (NLS) may undergo dephosphorylation by protein phosphatase 2C (PP2C). This dephosphorylation event is hypothesized to regulate the nuclear import of VirD2 (unpublished results by Y. Tao, P. Rao, and S. Gelvin). Additionally, VirD2 is likely to engage with CAK2M and TBP inside the nucleus. Given that both CAK2M and TBP form parts of the plant's RNA transcription apparatus, their engagement with VirD2 could aid in positioning the entire T-complex at the correct location for integration into the host chromosome [25].

Similarly, VirE2 interacts with VIP1, which is believed to be part of the plant's transcriptional machinery. Besides facilitating the nuclear import of VirE2, VIP1 might also contribute to the movement of the T-complex within the nucleus, directing it to chromosomal areas

where the host DNA is more accessible and thus more susceptible to T-DNA integration.

4. Manipulation of *Agrobacterium* for Genetic Engineering Purposes

According to the studies of late twentieth century, virulence-related strains of *Agrobacterium* comprise of large plasmids, although not all such strains are pathogenic. Genetic research revealed that a specific group of plasmids known as Ti (later Ri) plasmids caused tumour formation in plants [26]. Further analysis identified a segment of such plasmids, designated T-DNA, which was relocated and integrated into the plant's genome [27]. This realization prompted the idea of utilizing Ti plasmids as carriers to incorporate exogenous genes into plant cells.

Yet, the enormous size of the Ti plasmids and the scarcity of distinctive restriction sites within the T-DNA region presented challenges for the insertion of genes directly. To overcome this obstacle, researchers developed various strategies, categorized into two main approaches: (1) indirect cloning of the gene of interest onto the Ti plasmid, positioning it adjacent to the virulence genes on the same plasmid, and (2) cloning the gene into a separate replicon containing the T-DNA region, independent of the vir genes (T-DNA binary vectors).

Two primary strategies to introduce exogenous DNA onto the Ti plasmid were employed. One of them involved cloning of a specific DNA fragment (such as the T-region or the targeted region for modification) comprising distinctive restriction sites into versatile plasmids, like an IncPα-based vector, capable of replicating in both *Escherichia coli* and *Agrobacterium* [28]. Subsequently, the gene of choice, accompanied by a selectable marker for antibiotic resistance, was inserted into the distinctive restriction site of the target DNA region. As an alternate approach, transposon insertion could be used to introduce the resistance gene into the desired fragment [29] [30]. This engineered plasmid was then introduced into *Agrobacterium* via conjugation or transformation.

Verification of plasmid presence in *Agrobacterium* was achieved by selecting for resistance to antibiotics carried by both the plasmid backbone and the marker present nearby the desired gene. Following this, a second plasmid similar to that of the first one but containing a distinct marker for antibiotic resistance was integrated into the *Agrobacterium* strain comprising the initial plasmid. Selection of media containing both antibiotics allowed for the isolation of bacteria resistant to both, indicating either (i) cointegration of the first plasmid with the Ti plasmid, utilizing the latter's oriV for replication, or (ii) double homologous recombination (homogenotization) between homologous sequences flanking the gene of interest and resistance marker on both plasmids. In the first scenario, where the entire first plasmid cointegrates, the backbone

resistance marker would be expressed, leading to the identification and discarding of these bacteria. In the second scenario (homogenization), the plasmid backbone resistance marker would be lost.

An alternative method for presenting the exogenous DNA into the Ti plasmid's T-region leverages the incorporation of a ColE1 replicon, such as pBR322. This integration is first established within the T-region of a pre-existing Ti plasmid. Subsequently, the DNA intended for integration is introduced to a discrete molecule obtained from pBR322, including an additional marker for antibiotic resistance. The plasmid constructed is introduced into the modified *Agrobacterium* strain, followed by selection to withstand the effects of the new antibiotic. As ColE1 replicons are incompatible with *Agrobacterium*, stable expression of the plasmid-encoded resistance gene requires cointegration with the pBR322 segment already present in the altered T-region [31].

This technique has been further refined in the "split-end vector" system. Here, the desired gene is introduced into a vector derived from pBR322 by incorporating several key elements: (a) T-DNA right border (b) non - nptII chimeric gene for selection of transgenic plants (c) spectinomycin-streptomycin resistance marker for plasmid selection in *Agrobacterium* (d) region homologous to non-oncogenic section of an octopine type T-region. The fusion of this plasmid with the Ti-plasmid which lacks the right border but comprises the T-DNA homologous region enables the border activity and inserts the desired gene along with the

Cointegration of this plasmid with a Ti plasmid lacking a right border, but containing the T-DNA homologous region, restores border activity and integrates the gene of interest along with the plant selection marker inside the reconstructed T-region [32].

Each of these adjacent insertion strategies possesses several benefits and drawbacks. The initial method allows for targeting the exogenous gene to any desired location within the T-region (or other sections of the Ti plasmid). Yet, using such strategies is very difficult and requires advanced microbial genetic techniques, which discourages many laboratories from attempting it. While technically simpler, the second strategy restricts the cointegration of the foreign gene to pre-determined locations within the Ti plasmid where pBR322 was previously incorporated [33] [34]. Both approaches offer the benefit of maintaining the exogenous gene at such a minimal copy count compared to that of the Ti plasmid within *Agrobacterium*.

The inherent complexity of directly incorporating the alien genes into the T-region of Ti plasmids prompted certain laboratories to develop other strategies for using *Agrobacterium* to transfer genes to plants [35] [36]. Studies demonstrated that the T-region along with the vir genes can be successfully segregated into two distinct replicons.

However, when these replicons were present in the same *Agrobacterium* cell, the vir gene products were capable of performing their function across the T-region as well as processing the T-DNA and delivering it to the plant cells. This approach was named the "binary-vector system", where the replicon with the T-region is called the "binary-vector" whereas the replicon associated with vir genes is known as "vir helper".

The discovery of T-DNA binary vectors transformed the application of *Agrobacterium* in introducing genes into plants. This innovation empowered researchers lacking the technical skills required for modifying *Agrobacterium* for generating genetically altered plants. Such plasmids are favoured for their compact size and ease of manipulation in both *E. coli* and *Agrobacterium*, typically containing several distinctive restriction sites present in the T-region, enabling seamless cloning of genes of choice. Moreover, diverse carriers are constructed for specific objectives by incorporating various selectable markers for plants, promoters, poly (a) addition signals for transgene expression, enhancers to optimize expression levels, and signals directing protein localization within plant cells.

5. Applications of Ti plasmid

The fascinating ability of Ti plasmids to facilitate the transfer of T-DNA across kingdoms forms a cornerstone of *Agrobacterium tumefaciens'* pathogenesis. This bacterium can deliver any T-DNA-flanked DNA to a vast range of plant hosts, encompassing gymnosperms, dicotyledons, and even several agriculturally significant monocotyledons.

For plant biologists, *Agrobacterium*-mediated T-DNA transfer offers valuable tools beyond its role in pathogenesis. T-DNA tagging enables the isolation of novel plant genes through various methodologies. T-DNA's random integration into the genome serves as a powerful mutagen for identifying genes associated with distinct phenotypes. Moreover, if the mutagenic T-DNA contains a bacterial origin of replication, the altered gene can be easily isolated in bacteria. Alternatively, incorporating a selectable or scorable gene near one end allows the characterization of promoter activity when inserted downstream of a plant promoter. Conversely, an outward reading promoter within the T-DNA can modulate gene expression, potentially leading to interesting phenotypic effects.

While random T-DNA insertion benefits gene characterization, it poses a challenge for plant genetic engineering. T-DNA insertion into essential genes and flanking sequence rearrangements can have detrimental consequences. Ideally, T-DNA delivery would target specific sites in the plant genome. One promising approach utilizes the bacteriophage P1 Cre/lox system for site-specific integration. This system relies on the Cre recombinase to catalyze strand exchange between lox sites, achieving site-specific T-DNA integration upon co-expression with Cre in the plant.

Furthermore, the host range of *Agrobacterium* extends beyond plants, encompassing budding and fission yeast, filamentous fungi, and even human cells. It can efficiently deliver DNA to fungal protoplasts, conidia, and hyphal tissue, making it particularly valuable for species resistant to other transformation methods. This simple and efficient system opens up exciting possibilities for genetic manipulation and characterization of fungi, potentially translating existing plant research methodologies to fungal studies.

The recent discovery of *Agrobacterium*'s ability to transform HeLa cells further broadens its host range, raising intriguing questions about the potential of T-DNA transfer as a viable human gene delivery system.

Finally, while T-DNA is the primary interest for plant and biotechnology applications, Ti plasmid-encoded type IV secretion systems (T4SS) and other bacterial T4SSs can also translocate protein substrates. These systems hold promise for targeted delivery of therapeutic proteins to various eukaryotic cells, including human cells.

6. Future Prospects

Over the past two decades, the field of plant science research has witnessed a remarkable transformation with the development of genetic engineering technologies. One such technology, that has revolutionized modern agricultural biotechnology, is the use of *Agrobacterium* to genetically transform plants. Although *Agrobacterium* has a remarkably broad host range considering other plant pathogens and is highly efficient in the case of dicotyledons, its application in monocotyledons continues to be problematic [37]. Since two-thirds of the food for humankind comes from cereals [38], further development of effective agrotransformation techniques in leading cereals including rice, maize, wheat and barley, despite significant refinements thus far, is still required. Ti plasmid-mediated genetic modification allows for the transfer of multiple genes simultaneously. In the future, researchers may focus on stacking multiple genes for various traits (Trait Stacking), such as pest resistance, drought tolerance, and nutritional enhancements, to create crops with multiple beneficial characteristics. Scientists have already created a gene stacking system called GAANTRY (Gene Assembly in *Agrobacterium* by Nucleic acid Transfer using Recombinase technology) which has proven to be an efficient method of gene assembly and plant transformation that generates high-quality transgenic *Arabidopsis* and potato plants [39][40].

Genome editing tools like CRISPR-Cas9 and other advanced gene-editing technologies have gained prominence in recent years. These techniques enable precise modifications at specific locations in the plant genome without the need for introducing foreign DNA. In the future, the development of more precise gene-editing tools for plants may reduce the reliance

on Ti plasmid-mediated genetic modification. On the other hand, the isolation and engineering of new *Agrobacterium* strains using genome editing tools could also be a possibility. Specially designed bacterial strains may enhance the accuracy and effectiveness of gene editing and also improve the success rate of plant genetic transformations. The implementation of these approaches has the potential to revolutionize plant biotechnology, offering innovative methods for increasing crop yield and developing resistance against diseases.

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