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(REVIEW ARTICLE)

Agrobacterium tumefaciens: Biology and application in genetic engineering

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Abstract

Agrobacterium tumefaciens is a rod-shaped soil bacterium renowned for its unique ability to transfer tumour-inducing plasmid (Ti plasmid) segments to plant cells. This mechanism has been extensively exploited in plant genetic engineering. This review delves into the intricate biological interactions between *A. tumefaciens* and plant cells, including the critical steps of bacterial attachment, activation of virulence (Vir) genes, generation and transport of the T-complex, and integration of T-DNA into the plant chromosome. Furthermore, the review examines the engineering of *A. tumefaciens* as a transformation tool, focusing on the modifications of Ti plasmids to create binary and co-integrative vector systems, which have significantly improved the efficiency and versatility of transformation protocols. The paper also highlights applications of *Agrobacterium*-mediated transformation in the production of edible vaccines. By providing a detailed examination of the biological, technical, and practical aspects of *Agrobacterium*-mediated transformation, this review aims to offer insights into optimizing this technique for various plant biotechnology applications. Ultimately, understanding and improving *Agrobacterium*-mediated transformation is crucial for advancing plant biotechnology.

Keywords: *Agrobacterium tumefaciens;* Genetic engineering; plant transformation; Edible vaccine; Binary vectors

1. Introduction

Agrobacterium tumefaciens is a gram-negative rod-shaped soil bacterium that belongs to the Rhizobiaceae family. It is a plant pathogen that has the natural ability to transfer segments of its genetic material from its tumour-inducing plasmid (Ti plasmid) to plant cells [1, 2]. This ability has been harnessed for genetic engineering, facilitating the introduction of desirable characteristics into plant species. *Agrobacterium tumefaciens, originally noted for its role in crown gall formation,* has risen beyond its pathogenic roots to become a priceless tool in genetic engineering, attracting considerable attention.

An outstanding application of this organism is its use for plant transformation. It has been used for stable and transient transformation along with genome editing of plants [3]. *Agrobacterium*-mediated transformation has proven to be an effective tool for producing recombinant proteins and biopharmaceuticals such as edible vaccines, cytokines, industrial enzymes, blood components, dietary components, growth factors, and antibodies. It could also be used for studying fundamental plant genetics, gene function, and editing [4].

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It is the preferred and most effective method used for producing transgenic plants [5]. The benefits of this method include cost effectiveness, high reproducibility, and the possibility of transferring large DNA fragments. Genetically engineered drugs such as the CRISPR/Cas9 system, ZFNs, and TALENs can be delivered using this method [3].

2. Biological interactions between *A. tumefaciens* and plant cells

2.1. Attachment of *A. tumefaciens* to plant cells

When *A. tumefaciens* gets in contact with a plant, it causes an injury at the stem-root interface region and gains entry into the plant cell. Its attraction to the wounded plant occurs in response to signal molecules emitted by the plant cells, which increase exponentially in the wound sap and are attached to the walls of the plant cell. The attachment of *A. tumefaciens* to the cells of the plant is a necessary condition for the subsequent transfer of genomic material. The surface receptors of the plant cells and *A. tumefaciens* chromosomal gene (chvA, chvB, pscA, and aft) products are required for the attachment process. The chromosomal genes chvA, chvB, and pscA participate in the production, processing and export of cyclic β-1,2-glucan and other sugars. Apart from cyclic β-1,2-glucan and cellulose, *A. tumefaciens* synthesizes an extra exopolysaccharide, a unipolar polysaccharide that takes part in bacterial attachment [6, 7] (Figure 1).

2.2. Activation of virulence (Vir) genes

The Ti plasmid contains a 40 kb virulence region that carries approximately 25 genes encoding proteins that aid in the integration of T-DNA from bacteria into the plant host [8]. A group of virulence genes on the Ti plasmid contribute to this process. A minimum of eight operons, namely, virA, virB, virC, virD, virE, virF, virG, and virH, encode the virA, virB, virC, virD, virE, virF, virG, and virH proteins, respectively. Six of these operons (vir A, vir B, vir C, vir D, vir E, and vir G) are essential, while two (virF and virH) are nonessential [9].

Phenolic compounds secreted by the wound site of the host plant, such as acetosyringone, alpha-hydroxy acetosyringone, lignin, or flavonoid precursors, activate the expression of virulence genes. The most effective virulence genes are those encoding acetosyringone and other monocyclic phenolics. Other environmental conditions such as acidity, low phosphate, sugars and temperature are important for optimal virulence gene induction, but phenolics are the only required signal [10].

The two primary constitutive operons, vir A and vir G, encode for the VirA-VirG two-component system, which is responsible for triggering the transcription of other virulence genes. These host signalling compounds are identified by VirA in the periplasmic space of bacterial cells, where VirA functions as an autokinase. As a result, it becomes autophosphorylated by ATP, which in turn causes the positive regulatory protein, VirG to become phosphorylated [7]. VirA functions as a transcription factor, regulating the expression of virulence genes, when it phosphorylates VirG. When virulence genes are activated, a single-stranded (ss) T-DNA transfer complex is formed. Any sequence of DNA that lies between T-DNA borders will be sent as single-strand DNA to the plant cell and incorporated into the genome of the plant. VirD1 and VirD2 use the 25-bp border regions on the strand of T-bottom DNA as nicking sites. Double-stranded T-DNA is unwound by the site-specific helicase VirD1. To create single-stranded linear DNA known as the T-strand, VirD2 acts as a nuclease and cuts the bottom strand of T-DNA from the left and right borders [5]. VirD2 remains covalently attached to the 5’ end of the ssT-strand and forms the virD2/T-strand complex. This association protects the 5' end of the ss-T-strand from exonucleolytic attack, which also identifies the 5' end as the leading end of the T-DNA transfer complex.

The overdrive enhancer element is recognized by and bound to the Vir-C1 and Vir-C2 proteins. VirE2, a single-strand DNA binding protein coats the ssT-DNA-VirD2 complex thereby preventing attack from nucleases. The VirD2 operon contains two plant nuclear location signals, while VirD2 contains one nuclear location signal that transports the VirD2/T-strand complex from bacteria to the host plant nucleus. The VirD2/T-strand complex leaves bacterial cells through the Ti-pilus, referred to as the type IV secretion system (T4SS), after passing through a rod-like structure. The two operons, virB and virD4, combine to generate this type IV secretion system (T4SS). The purpose of the virB operon is to create an appropriate cell surface structure for the transfer of the ssT-DNA complex from bacteria to plants. The transfer of ss-T-DNA from the Ti plasmid to the plant cell also requires the VirD4 protein. VirD4 is primarily involved in signalling ATP-dependent interactions with the protein complex necessary for T-DNA translocation. The octopine Ti plasmid has two additional vir operons, namely, virF and virH. A 23 kDa protein is encoded by the virF operon. The virH operon is composed of two genes that encode the proteins VirH1 and VirH2. Although these virulence proteins are not needed, they can increase the effectiveness of the transfer by detoxifying certain plant compounds that may hinder the growth of bacteria. Additionally, they are essential for the host range specialization of bacterial strains for particular plant species [5].

2.3. Generation and transportation of the T-complex

The T-DNA is flanked by 25-bp border sequences. The VirD2 protein, which participates in the processing and transfer of T-DNA with the help of VirDl, VirCl and VirD3, pinpoints the border repeats and makes a nick in the middle of the third and fourth bases at the T-DNA bottom strand. The VirD2 protein attaches covalently to the 5' end of the bottom strand, after which the bottom strand is released from the Ti plasmid to form the T-strand. The T-strand is coated with the VirE2 protein to produce the T-complex. The VirB and VirD4 proteins create a channel between *A. tumefaciens* and the plant cell through which the T-complex travels into the plant cytoplasm. VirD2 and VirE2 house the nuclear localization signals that mediate T-complex transfer from the plant cytoplasm nucleus (Figure 1).

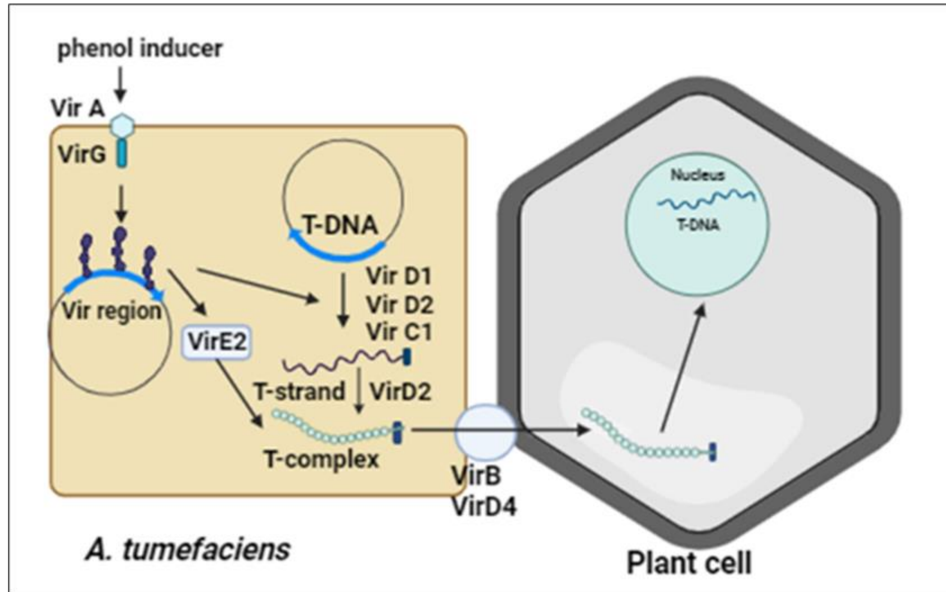

Figure 1 Interaction between *Agrobacterium tumefaciens* and plant cells

2.4. Integration of T-DNA into the plant chromosome

After the T-complex enters the plant nucleus, several T-strands become double-stranded DNA. The integration of T-strands into the host chromosome is facilitated by the host enzymes and components of the DNA replication machinery. The fungal transformation system was used to demonstrate that VirD2 participates in the protection of the integrity of the right border, VirE2 ensures that the left end of the T-DNA is protected, and VirC2 guarantees correct processing.

2.5. Expression of T-DNA in plant hosts

Figure 2 Crown gall

Bacterial DNA is expressed in a wide variety of ways in certain plant species, or it can remain silent within the genome of those species. The genes that the T-plasmid encodes are expressed in plants. Phytohormones such as auxin, cytokinin and opines (amino acid derivatives) are synthesized by enzymes encoded by T-DNA. When large amounts of phytohormones are produced, the outcome is neoplastic growth known as crown gall (Figure 2) [5].

3. *Agrobacterium*-Mediated Transformation

Genetic engineering has been used to modify plants so that they can be used as systems for the production of recombinant proteins and biopharmaceuticals such as edible vaccines, cytokines, industrial enzymes, blood components, dietary components, growth factors and antibodies [4]. It has also been used to produce plants that are more productive, resistant to pests, viruses, and herbicides, and tolerant to cold, salt, and drought [11]. *A. tumefaciens* has been widely employed in plant genetic engineering [12]. *Agrobacterium tumefaciens* genetically transforms a wide range of host cells, mostly dicotyledons, some gymnosperms and a few monocotyledon plants. It also transforms non-plant organisms such as human cells, sea urchin embryos, fungi, algae and *Streptomyces lividans* [13, 14].

The *Agrobacterium*-mediated transformation approach has been used in basic biology research to evaluate the function of proteins or the regulation of genes in transgenic plants. The benefits of *Agrobacterium*-mediated transformation include cost effectiveness, high reproducibility, ease of use, well-defined DNA insertion into the host chromosome and the possibility of transferring large DNA fragments [15]. Conventional transformation methods such as protoplast transformation by microinjection, electroporation or polyethene glycol fusion are not suitable for producing transgenic plants, as regenerating plants from protoplasts is time-consuming and of low efficiency [16]. In microprojectile bombardment, the bombardment process can lead to the loss of DNA molecular integrity and can cause multiple-copy DNA insertions. This results in epigenetic silencing of the transgene in the transgenic plant or genetic instability [17]. Due to the disadvantages of chemical and physical transformation methods, *Agrobacterium tumefaciens*-mediated transformation is preferred and mostly used for generating transgenic plants.

3.1. Types of *Agrobacterium-Mediated* Transformation

There are two forms of *Agrobacterium-mediated* transformation: transient and stable transformation. In transient transformation, T-DNA does not integrate into the host genome, and its expression usually lasts for a short time [18]. An advantage of this approach is that the results of the experiment can be observed in a short time. Stable transformation involves the integration of T-DNA into the host genome [19]. It is an extensive process that requires the establishment of tissue culture to support the growth of a whole plant from transformed cells or tissues.

3.2. Engineering *Agrobacterium tumefaciens* as a Tool for Transformation

After the mechanism by which *Agrobacterium* transforms its host was established, attention was given to its potential as a tool for genetic transformation. For *Agrobacterium tumefaciens* to be suitable for transformation, its wild type needs to be engineered. Some of the natural characteristics of the Ti plasmid have to be completely removed, e.g., opine biosynthesis in planta and the genes that cause tumour formation. The features of some transformation machinery components had to be supplemented and enhanced, e.g., the reduction of the size of the Ti plasmid to fit with what standard molecular cloning can handle. The size of the wild-type Ti-plasmid (200 kb) was reduced to less than 10 kb (binary vectors), resulting in increased transformation efficiency [20].

3.2.1. Modification of tumour-inducing (Ti) plasmids

Ti plasmid is a circular DNA molecule of 200 kb that is present in pathogenic species of *Agrobacterium tumefaciens.* The transfer DNA (T-DNA) of the Ti plasmid comprises eight genes which are expressed in plants, and are responsible for crown gall formation in plants. Structurally, it contains distinct regions with specific functions, including, transfer DNA (T-DNA), sequence border, virulence region (vir region), origin of replication, tra genes and genes for opine synthesis (Figure 3) [8]. The right and left sequence borders, which are 25 bp repeat sequence limits on either end, define the highly conserved T-DNA segment, which is 23 kb or 15–40 kb in length. The right sequence boundary marks the beginning of the T-DNA transfer whereas the left sequence boundary marks the end. T-DNA is composed of two different gene types: (1) Oncogenic genes and enzymes that encode genes for producing auxins and cytokinins. (2) The genes that code for the production of opines. Most *Agrobacterium* strains produce an octopine or a nanopine form of opine [5].

Ti plasmids are typically employed as natural vectors for plant cell genetic engineering. However, wild-type Ti plasmids are not necessarily the best choices for general gene carriers since they encourage the disorderly proliferation of recipient plant cells because of oncogenes in the Ti plasmid's T-DNA. Naturally occurring Ti plasmids are not suitable

for use in transformation experiments. Therefore, considerable modifications are required to make these plasmids appropriate for plant transformation [8]. By altering the Ti plasmid, three distinct vector types can be created:

- Disarmed Ti plasmid
- Intermediate vectors
- Helper vectors

Disarmed Ti plasmid

Disarming occurs when genes involved in oncogenesis (e.g., auxin and cytokinin production) and opine synthesis are removed from T-DNA regions. As a result, T-DNA loses its capacity to form tumours, although the transfer and integration of T-DNA are not affected [8]. In disarmed Ti plasmids, exogenous DNA occupies the position of oncogenes in the T-DNA region. Examples of disarmed Ti plasmid vectors include the following:

a) SEV series: the right border of the T-DNA and the phytohormone genes coding for auxin and cytokinin, are replaced by the bacterial kanamycin resistance gene while the left border and a small portion of the original T-left DNA segment (TL) known as the left inside homology (LIH) remain intact.

b) pGV series: A section of the pBR322 vector sequence is used in place of the phytohormone genes. Both the left and right border sequences and the nopaline synthase gene of the Ti plasmid are conserved.

The first outstanding breakthrough in the establishment of a transformation vector system for *Agrobacterium*-mediated transformation was the creation of disarmed *A. tumefaciens* strains by eradicating the wild-type T-DNA from the Ti-plasmid [21].

Intermediate vectors

These are small plasmids (*E. coli* vectors) based on pBR322 that have a T-DNA region. intermediate vectors can proliferate in *E. coli* and be conjugated into *Agrobacterium*. However, because they cannot reproduce in *A. tumefaciens,* they carry DNA segments that are identical to the disarmed T-DNA, allowing recombination to form a cointegrated T-DNA structure [22].

Helper vectors

These are small plasmids maintained in *E. coli,* containing transfer (tra) and mobilization (mob), which allow conjugative deficient intermediate vectors to be transferred into *Agrobacterium* [22].

These three modified *Agrobacterium* Ti plasmids can generate two major plant vectors: co-integrative and binary.

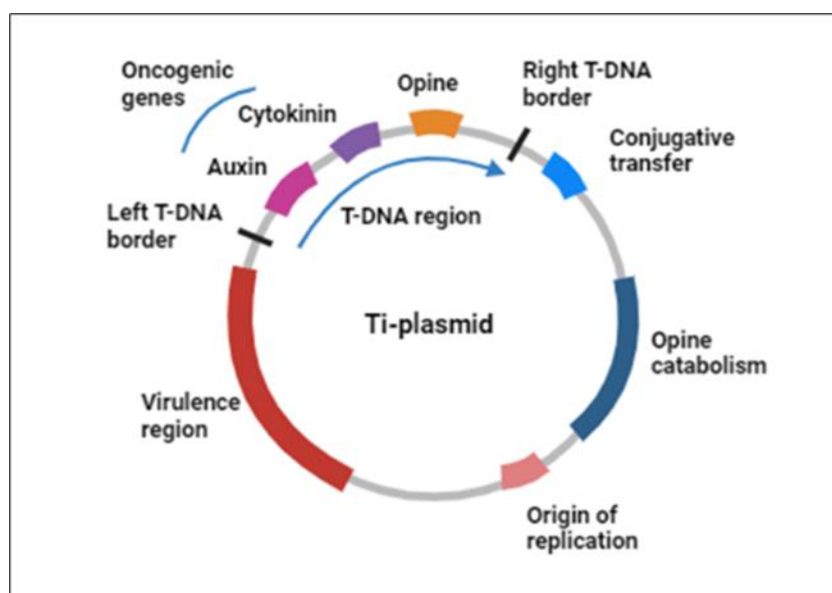

Figure 3 Natural Ti-plasmid

3.2.2. Binary vectors

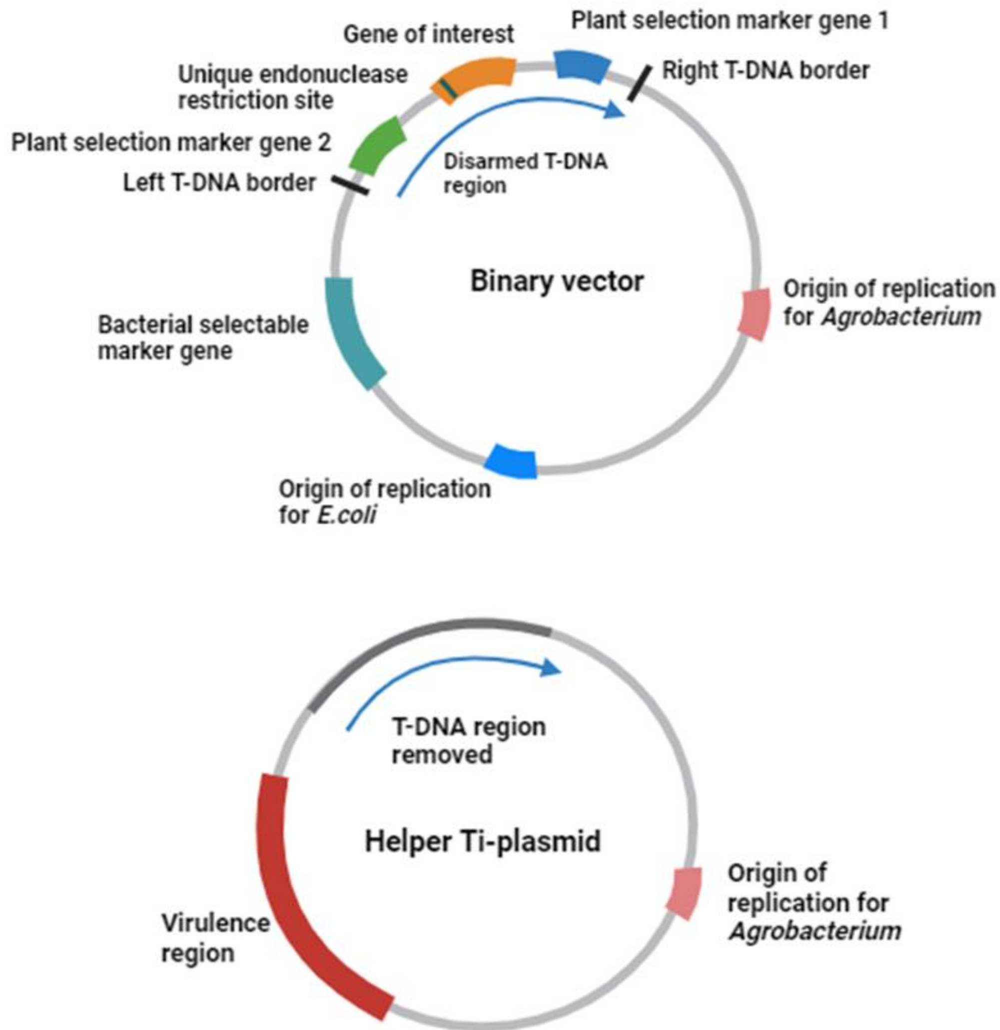

Figure 4 Binary vector

A binary vector consists of two plasmids, a helper plasmid and a disarmed Ti plasmid. PAL4404 is an example of a helper Ti plasmid, and BIN is an example of a disarmed Ti plasmid. The helper plasmid contains only the virulent region. Binary vectors catalyze the insertion of transgenes from *Agrobacterium* into plants [5]. The binary transformation vector system utilizes the fact that T-DNA and the virulence genes required for T-DNA transfer can be on different replicons; T-DNA is carried on a broad-host range replicon while the virulence gene is located on the disarmed Ti-plasmid [20]. Small plasmids with the ability to replicate in both *E. coli* and *Agrobacterium* are used. Engineered T-DNA is inserted into the plasmid and introduced into the disarmed *Agrobacterium* strains. The cis-elements that are essential for T-DNA transfer are the two 25-28 base pair direct repeat borders. Therefore, the wild-type natural opine synthase gene and oncogenes of the T-DNA are replaced with the gene of interest, which allows any DNA positioned between the borders to be transferred to the host cell. However, since T-DNA does not mediate its transfer, the virulence gene, which is located in the virulence region and is required for T-DNA transfer and integration, is modified. The modification of their regulation and copy number has proven to be advantageous for increasing the efficiency of transformation. Thus, the size of the T-DNA that can be inserted into the plant can be enlarged. The ability of virulence genes to act in trans has resulted in the development of binary and super-binary transformation vectors to increase the range of species that can be transformed by *Agrobacterium* [21].

With the development of recombinant DNA technology, T-DNA binary vectors have advanced over the years to be widely developed and specialized for different purposes [23]. T-DNA-based binary vectors comprise numerous features,

including (1) a T-DNA right and left border repeat sequence, which defines the T-DNA region; (2) restriction endonuclease sites within the T-DNA to allow the insertion of one or more gene(s) of interest; (3) Selectable marker genes to function in bacteria (*E. coli and A. tumefaciens*) and in plants; and (4) the origin(s) of replication which allows plasmid replicates in bacteria (Figure 4).

3.2.3. Co-integrative vectors (hybrid Ti plasmids)

A co-integrative vector is created by homologous recombination between two plasmids [8]. For instance, PTiC58 (a nopaline-type Ti plasmid) was rendered ineffective by substituting the pBR322 sequences of *E. coli* plasmid for its oncogene. This resulted in the production of a co-integrative vector, called pGV3850. Except for the T-DNA region, every region seen on a typical Ti plasmid, including the vir region and the right and left border sequences, is present in this pGV3850 disarmed Ti plasmid. Although cointegrated vectors were among the first modified and engineered Ti plasmids created for *Agrobacterium*-mediated transformation, they are no longer widely used.

4. Application of *Agrobacterium*-Mediated Transformation in Edible Vaccine Production

Vaccination has been proven to be the most efficacious means of combating infectious diseases in our world today. Notwithstanding, most people that live in developing countries are unable to afford it because of its high cost. The complexity of conventional vaccine production which includes; the high cost of fermentation and purification systems, cold chain systems, transportation and sterile delivery account for the high cost of conventional vaccines. Conventional vaccine dependence on cold chain systems is one of its major limitations. Cold chain is a low temperature-controlled system that guarantees correct storage and dispersal of vaccines from the manufacturing company to the patient. Conventional vaccines are distributed through the cold chain at 2–8 °C for the best output. Alteration of the cold chain system leads to the denaturation of the vaccine and subsequently, incompetent vaccine activity and inimical effects after administration. These challenges of conventional vaccines necessitated the investigation of edible vaccines which is an alternative procedure for the production and delivery of vaccines [24].

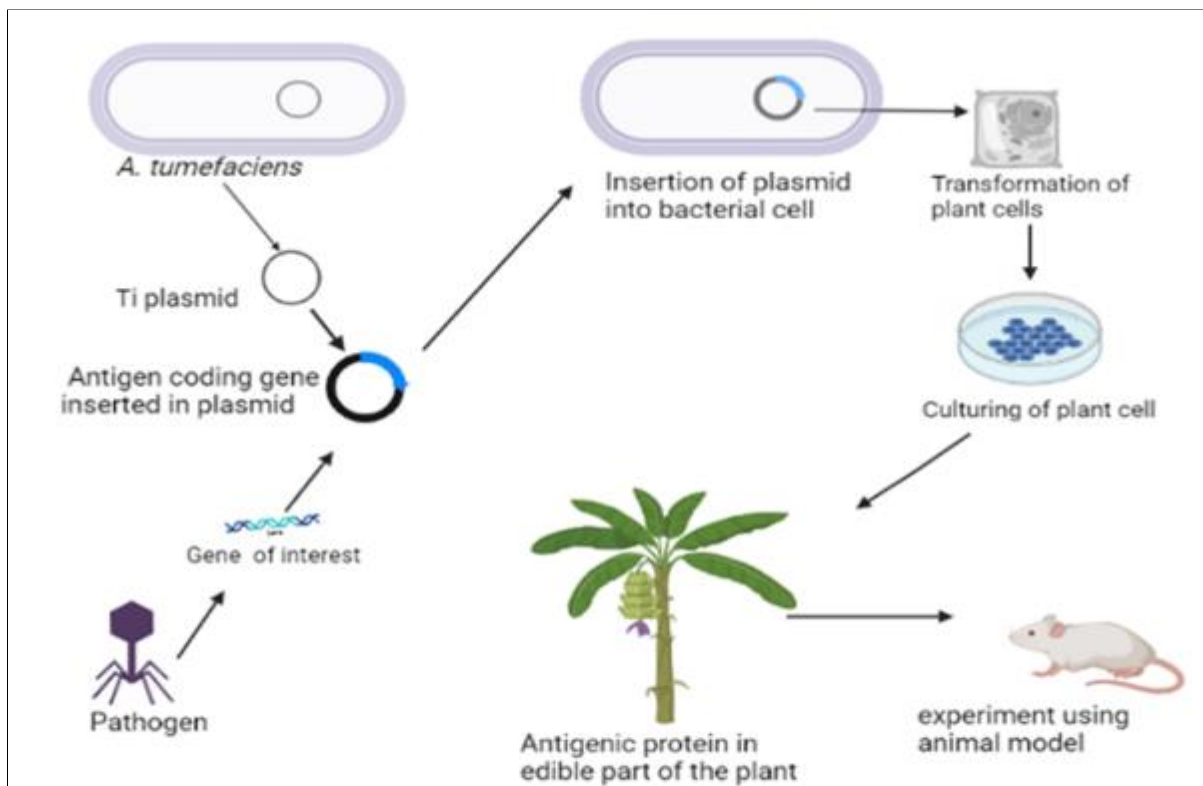

Figure 5 Production of edible vaccine using *Agrobacterium tumefaciens*

Edible vaccines are subunit vaccines made by infusing antigenic peptides into plant edible parts, which stimulate immune response upon oral administration. There are vaccines produced in genetically modified plants and consumed orally. The first step in the production of edible vaccines is to isolate and purify the gene that encodes an antigenic structure. The gene is then transformed into the plant using *Agrobacterium tumefaciens*. The transformed plants are

subsequently grown in vitro (Figure 5). For transformed cells to be identified, the foreign gene that is integrated into the plant chromosome must be detected. For this to happen, some antibiotics and herbicide resistance genes are inserted into the T-DNA region of the binary vector and used as selection marker genes. The most widely used selection marker gene systems in plants include hygromycin phosphotransferase (HPT) for hygromycin resistance and the neomycin phosphotransferase (NPTII) gene for kanamycin resistance [25].

4.1. Plant-Based Human Vaccines

4.1.1. Hepatitis B vaccines

Globally, a million people die from liver cancer as a result of hepatitis B virus (HBV) infection. Given that HBV can be spread through sexual intercourse and by vertical transmission from infected mothers to their babies, the production of vaccines to prevent infections is of utmost importance. In addition to the systemic defence offered by the injectable yeast-derived HBsAg subunit vaccine, mucosal immunity can be achieved by the ingestion of transgenic plants that express recombinant HBsAg (rHBsAg). Over the past 20 years, a significant amount of research has shown that HBsAg is expressed in a range of plant systems, including corn, banana, potato, lettuce, tomato, and tomatillos [26, 27, 28]. HBsAg expression was first detected in transgenic tobacco and potato tubers. These plants were later utilized to show mouse oral immunogenicity [29].

4.1.2. Human immunodeficiency virus (HIV) vaccines

HIV is an infectious virus that targets CD4+ lymphocytes and eventually causes the loss of adaptive immune system activities. It is currently a well-known and feared pathogen. The outcomes of the enormous efforts made by researchers worldwide over the past 30 years to create a vaccine are, at best, mediocre. Plants have been used in several studies to express recombinant HIV antigens. Scientists generally agree that plant-based vaccines induce significant and protective mucosal immune responses. They thus frequently concentrate on antigen delivery at mucosal locations. Virus-like particles (VLPs) have been used in numerous plant-based attempts to develop HIV vaccines to boost antigen density and produce a repeating antigen array that can more successfully bind to B-cell receptors. The use of potato virus X (PVX) to create chimeric plant virus particles displaying the highly conserved "ELDKWA" epitope from the HIV glycoprotein gp41 is an intriguing early example [30]. The recombinant virus utilized to infect *N. benthamiana* plants was fused with the epitope coding region to the N-terminus of the PVX coat protein, resulting in a robust generation of chimeric viral particles. Mice were immunized with the purified virus intraperitoneally (IP) without adding an adjuvant, producing serum IgG and IgA specific to ELDKWA.

5. Conclusion

Agrobacterium-mediated transformation remains a pivotal technique in plant genetic engineering, offering a versatile and efficient method for introducing new traits into plants. Advances in understanding the molecular mechanisms of T-DNA transfer and integration, coupled with improvements in vector design and transformation protocols, have significantly enhanced the efficiency and scope of this technology. Continued research into the interaction between *Agrobacterium* and host plants, as well as developing novel transformation methods, will further expand the potential applications of this powerful tool. As the demand for recombinant proteins grows, *Agrobacterium*-mediated transformation will undoubtedly play a crucial role in meeting global biotechnological challenges.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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