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A Review: Agrobacterium-mediated gene transformation to increase plant productivity

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ABSTRACT

In genetics and molecular biology, Gene transformation is a gene alteration technique that involves the introduction and expression of a foreign gene into the host organism. There are many gene transformation methods like particle bombardment, electroporation, micro-injection, PEG for different biotechnological experiments. But Plant gene transformation is a widely used procedure for obtaining transgenic plants and plant models to understand gene functions. Agrobacterium tumefaciens is a natural genetic engineer which is rod-shaped, gram-negative soil-born bacteria. Initially, Agrobacterium was utilized to transform only dicot plants but over the year's modification in plant transformation protocol it was now utilized in monocot plants as well as in fruits plants too. Agrobacterium tumefaciens inserts its DNA (Transfer DNA-T-DNA) into the host plant. The transmitted DNA is randomly integrated into the host cell's genetic material inside the infected plant cell nucleus. Alternatively, bacterial DNA can transiently remain in the nucleus without integrating into the genome, but it still replicates alongside the plant genome, using its machinery and expressing its genes to make separate gene products. Besides the traditional method, new research has also been done to transform the plants through agrobacterium. Various methods have been developed to transform monocotyledonous plants such as wheat, maize, rice, and fruity plants. Generally, dicotyledonous plants can be transformed by the traditional method of agrobacterium but various methods have also been developed for dicots for various applications. Here, we have taken an example of a tobacco plant (nicotiana tabacum) transformed with different methods.

Keywords: *Agrobacterium tumefaciens*, Gene Transformation, *Nicotiana tabacum*, T-DNA, Ti Plasmid.

INTRODUCTION

Nowadays, genetic transformation is a common approach in the genetic engineering field. Genetic transformation is a technique that involves the introduction and expression of foreign genes into the host organism [1]. Several attempts to transfer DNA into plant tissues were reported in the 1960s and 1970s, but no stable transformation was ever proven [2]. There are different six types of genetic transformation methods that have been demonstrated including physical gene transformation, chemical gene transformation, biological gene transformation method, Vectors based on naturally occurring plasmids of Agrobacterium, Direct gene transfer methods using various types of plasmid DNA, Vectors based on plant viruses. These methods are not so preferable as they have more disadvantages such as the specific requirement of plant protoplast culture as to make protoplast culture is own technical method for transformation and the transformation of genes at the specific site in the host genome. However, virus-mediated gene transformation is not a stable transformation as it can't pass the transient expression in the next generation. Transient expression of a gene or stable transformation can be determined by the status of integration of transgene in the host plant genome, All the above-mentioned methods are amenable for transient expression as well as a stable transformation but the Agrobacterium-mediated genetic transformation method is more specific for stable transformation [3].

This Agrobacterium-mediated genetic transformation process is an example of horizontal gene transfer (HGT), also known as lateral gene transfer, which refers to the movement of genetic information between organisms that are more or less distantly related [3]. There are main two Agrobacterium strains are known for the gene transformation method which are Agrobacterium tumefaciens and Agrobacterium rhizogenes. Both the strains have their plasmids known as Ti plasmid (tumour inducing) and RI plasmid (root inducing) respectively. The transgenic tobacco plants were generated first by transformation using the soil bacterium Agrobacterium tumefaciens [4]. HGT from bacteria to plants has been limited to Agrobacterium rhizogenes, whereas the related bacterium Agrobacterium tumefaciens changes a wide range of host plants by transferring a piece of the enormous tumor-inducing plasmid known as T-DNA into host cells [5].

Introduction of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a rod-shaped, gram-negative soil bacterium from the Rhizobiaceae family that causes crown gall disease in over 140 dicotyledonous plant species [6]. Crown gall disease is caused by *Agrobacterium tumefaciens*, and hairy root disease is caused by *Agrobacterium rhizogenes*. We concentrated on gene transformation using *Agrobacterium tumefaciens* bacteria in this study.

Ti plasmid

During the examination of the pathogenic strains of A. tumefaciens, one or several homologs large (±140–235 kbp in size) supercoiled circular plasmids were isolated [7]. Based on its tumour-inducing nature in host plants, the plasmid was called "tumour-inducing (Ti)-plasmid" [7,8]. This is a big plasmid (more than 200 kb) that contains multiple genes involved in the infection process [6]. The most remarkable feature of the Ti plasmid is that the T-DNA contains eight genes that are expressed in the plant cell and are responsible for the cancerous properties of the transformed cells. This Ti plasmid can develop in tissue culture indefinitely as a callus even in the absence of phytohormones, which are required for normal cell growth *in vitro* [6]. Ti plasmid has different regions with different functions. It has T-DNA (20kb), sequence borders, vir region (virulence region), ori c region (origin of replication), tra genes, and genes for opine synthesis.

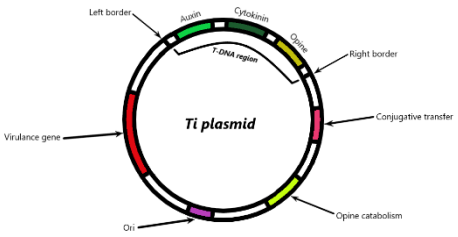

Figure 1: Structure of Ti plasmid

Organization of T-DNA

T-DNA (23kb or 15-40kb) is a highly conserved DNA segment defined by 25 bp repeat sequence boundaries on either end known as the right and left sequence borders. Generally, T-DNA transfer begins with the right sequence boundary and ends with the left sequence border. T-DNA is kept in a stable state in plant cells after transformation, and it is subsequently passed on to daughter cells as an important part of the chromosomes. T-DNA is made up of two types of genes: 1. Oncogenic genes and enzyme-encoding genes are involved in the manufacture of auxins and cytokinins and are responsible for tumour formation. 2**.** the genes encoding for the synthesis of opines [9,10]. Most common Agrobacterium strains produce an octopine or a nopaline form of opines [11].

Vir region

The Ti plasmid contains a 40 kb Vir region which carries approximately different 25 genes that code for the proteins amenable to excision, movement, and integration of T-DNA [6]. A group of virulent genes found on Ti-plasmid aid in the mechanism of T-DNA transfer from bacteria to host plant. Approximately 25 virulent genes grouped into at least 8 operons, virA, virB, virC, virD, virE, virF, virG, and virH, encoding VirA, VirB, VirC, VirD, VirE, VirF, VirG, and VirH protein, respectively [12,13]. This vir region present in the Ti plasmid does not itself integrated with the host plant genome but it helps the T-DNA to transfer from bacteria to plant cells and integrate with the plant genome. Vir gene expression is induced by phenolic substances released from the wound site of the host plant, such as acetosyringone, alpha-hydroxy acetosyringone, and lignin or flavonoid precursors [14]. Acetosyringone and other monocyclic phenolics are the most potent vir gene inducers [15]. The function and role of all vir genes playing a crucial role in Agrobacterium-mediated transformation is given in table Below:

Table 1: Role or vir region of T-DNA [6]

Operon/ Genes	No. of Genes	Functions
vir-A	1	Get activated by phenolic compounds (acetosyringone), Encodes a sensor protein, functions as auto kinase (phosphorylates itself and vir G)
vir-B	11	Membrane protein; formation of conjugal tube (Type IV secretion system) for T-DNA transport. Vir-B11 has an ATPase activity
vir-C	2	Act as an enzyme Helicase, involve in the unwinding of DNA
vir-D	4	Vir-D1 has topoisomerase activity, virD2 has endonuclease activity, vir D4 serves as linker for the formation of conjugal tube
vir-E	2	Vir E2 act as SSB protein binds to T-DNA during its transfer and protect it against exonuclease activity
vir-F	1	Not well understood
vir-G	1	Dimerises itself by vir A activity, DNA binding protein induces expression of all other Operon
vir-H	2	Not well understood

Ori C (origin of replication)

This region on the Ti plasmid is responsible for the start of replication of the own genome.

Mechanism of Agrobacterium-mediated genetic transformation

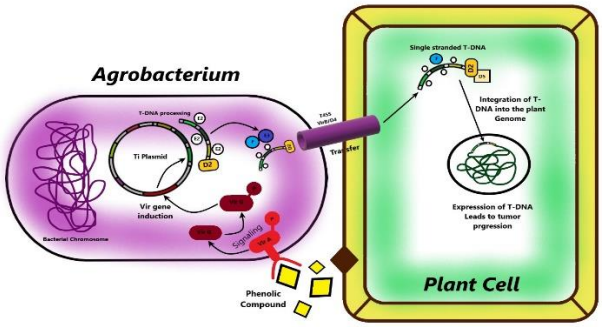

Figure 2: Mechanism of Agrobacterium-mediated genetic transformation

Agrobacterium-mediated gene transformation generally mimics the mechanism of natural gene transformation. The research of natural plant transformation began when some dicotyledonous plants showed fleshy rough roundish surface morphology on the crown of the roots (region joining root and shoot)[16]. During the investigation of this tumour-like outgrowth of root crown tissue, *Agrobacterium tumefaciens* bacteria were present there [17]. When these Gram-negative soil bacteria were introduced into injured young tissues of healthy plants, secondary tumours were formed. This recently formed secondary tumour is indistinguishable from crown gall tumours [16]. After this, the scientists have derived the explant from the interior of secondary tumour which continuously proliferates in auxin and cytokinin lacking medium and synthesized unusual amino acid derivatives such as guanido amino acids, octopine N2-(D-1-carboxyethyl)-L-arginine and nopaline N2-(1,3-dicarboxypropyl)-L-arginine[18,19]. Bacterial isolation from secondary tumour cultures revealed that no one of these cultures has yielded any growth of A. tumefaciens[20]. After this, scientists observed that the volume of the secondary tumour was still increased even after bacteria were absent, which indicates that bacteria only trigger tumorigenesis, not involved in the whole process[20]. It was subsequently shown that the undifferentiated callus can be cultivated in vitro even if the bacteria are killed with antibiotics, and yet retains their tumorous properties[6].

The ability to cause crown gall disease is associated with the presence of the Ti plasmid within the bacterial cell[6].

Scientists have proposed that when plants stem get wounded, it will release chemicals such as organic acid compounds (pH 5.0–5.8) as routine secreted chemical and phenolic compounds such as acetocyronigen and alpha-hydroxy acetocyronigen[21]. These phenolic compounds are then recognized by the separate region of Ti plasmid known as vir (virulence) region. This vir region is consist of a\ six essential operons (vir A, vir B, vir C, vir D, vir E, virG) and two non-essential (virF, virH). Notably, phenolic chemicals like acetosyringone do not draw bacteria to injured plant cells. Instead, the bacteria appear to respond to basic compounds like carbohydrates and amino acids, and the vir genes are activated following attachment[22,23]. Vir A and vir G are the main two constitutive operons that codes for a two-component (VirA-VirG) system activating the transcription of the other vir genes[26]. These host signal compounds are recognized by VirA on the periplasmic space of bacterial cells which acts as autokinase and therefore get autophosphorylated itself by ATP and thereby trigger phosphorylation of positive regulatory protein VirG[24,25,27]. When VirA phosphorylates VirG, it acts as a transcriptional factor, controlling the expression of vir genes[28,29]. The activation of vir genes produces the formation of a single-stranded (ss) T-DNA transfer complex. Any DNA sequence which is placed between T-DNA borders will be transferred to the plant cell as single-strand DNA and integrated into the plant genome. The 25-bp border regions on the T-bottom DNA's strand serve as a nicking location for VirD1 and VirD2. The site-specific helicase VirD1 unwinds double-stranded T-DNA. VirD2 functions as a nuclease, cutting the bottom strand of T-DNA from the right and left borders to form single-stranded linear DNA known as T-strand[30,31]. VirD2 remains covalently attached to the 5'-end of the ssT-strand and forms the virD2/Tstrand complex. This association prevents the exonucleolytic attack to the 5'-end of the ss-T-strand[32] and distinguishes the 5'-end as the leading end of the T-DNA transfer complex. Vir-C1 and Vir-C2 proteins recognize and bind to the overdrive enhancer element. Recent research suggests that the type of T-DNA intermediate produced (single- or double-stranded) depends on the Ti plasmid, with nopaline plasmids preferring double-stranded T-DNA and octopine and succinopine plasmids preferring single 'T-strands,' where the T-DNA is split into non-contiguous sections[33]. This ssT-DNA-VirD2 complex is coated by the 69 kDa VirE2 protein, a single strand DNA binding protein. This cooperative association of virE2 protein prevents the attack of nucleases. VirE2 contains two plant nuclear location signals (NLS) where VirD2 operon contains one NLS Which is specifically used to transfer VirD2/T-strand complex from bacteria to the host plant nucleus[34]. VirD2/T-strand complex passes through a rodlike structure and exits from the bacterial cells through Ti-pilus which is known as type IV secretion system (T4SS)[35]. This type IV secretion system (T4SS) forms with the two operons, virB and virD4. 9.5 kb virB operon has a function for the generation of a suitable cell surface structure for the ssT-DNA complex transfer from bacterium to plant[36]. The VirD4 protein is also required for the transportation of ss-T-DNA from the Ti plasmid to the plant cell. The primary function of VirD4 is to provide a signal for the ATP-dependent connection with the protein complex required for T-DNA translocation. Two accessory vir operons which present in the octopine Ti plasmid, are virF and virH.

1. The virF operon encodes for a 23 kDa protein. When the T-DNA complex is transferred to the plant cells via the conjugal channel or independently, it functions as VirE2 export from the bacterial cell to plant cell[9].

2. The virH operon is made up of two genes that code for the proteins VirH1 and VirH2. These Vir proteins are not required, but they can improve transfer efficiency by detoxifying some plant chemicals that can interfere with bacterial growth. They also play a crucial role in bacterial strain host range specialization for distinct plant species[37].

Integration of T-DNA into Plant Genome

The molecular process of T-DNA integration into the host plant genome is currently unknown. T-DNA enters a plant cell as a single-stranded strand. In the nucleus, it is instantly transformed into a double-stranded form. T-DNA integration occurs at random locations throughout the plant genome, not preferentially at hypomethylated regions of transcriptionally active sites. Two Vir proteins are important in this integration step: VirD2 and VirE2, which are the most important, and probably VirF has played a minor role in this process[9]. Generally, T-DNA integration is accompanied by short deletion of 23-79bp at the site of recombination or the target site[6].

Expression of T-DNA in the plant host cell

The expression of the bacterial DNA into plants depends on the plant species. In some plant species, bacterial DNA is expressed in a broad range but in some plant species, bacterial DNA cannot express itself or it can be silent in the genome. When the bacterial T-DNA expresses in the plant cell, it causes the formation of tumour growth at the site of the crown in plants due to excessive production of auxin, cytokinin and opines in the plant cell. By this method, scientists have proposed that we can transfer our gene of interest in plants by substituting the T-DNA in Ti plasmid.

Modification in Ti plasmid

Generally, Ti plasmid is used as a natural vector for genetic engineering in the plant cell. However, due to the presence of oncogenes in the Ti plasmid's T-DNA, wild-type Ti plasmids are not always ideal as general gene carriers since they promote the disordered proliferation of recipient plant cells. T-oncogenic DNA's action hinders the capacity of plant cells to regenerate and complete the plant[6].

Naturally occurring Ti or Ri plasmids are not suitable for the transformation experiment. So, making these plasmids amenable for plant transformation needs some modification[6]. Different three types of vectors can be formed by modifying the Ti plasmid:

1. Disarmed Agrobacterium Ti plasmid
2. Intermediate vectors
3. Helper vectors

1. Disarmed Agrobacterium Ti plasmid

When the genes responsible for oncogenesis (auxins and cytokinin production) and opine synthesis were deleted from T-DNA regions called disarming[6].

In these Ti plasmids, the oncogenes located in the T-DNA region have been replaced by exogenous DNA. The deletion of these genes loses the tumor formation capability of T-DNA but does not affect T-DNA transfer and integration[6]. Selectable marker genes are sometimes introduced into T-DNA to distinguish transformed cells from normal ones. Herbicide resistance markers that are often utilized include phosphinothricin, chlorsulfuron, sulfonamide, and glyphosate. Inserting bacterial selectable markers such as trimethoprim, streptomycin, spectinomycin, sulfonamides, bleomycin, hygromycin, kanamycin, neomycin, or gentamicin analyses the absorption of a modified plasmid into a bacterial cell. There are some examples of these vectors:

a) SEV series: the right border of the T-DNA, as well as the phytohormone genes coding for cytokinin and auxin, are removed and replaced by a bacterial kanamycin resistance gene, while the left border and a small portion of the original T-left DNA's segment (TL) are left intact (referred to as Left Inside Homology (LIH)).

b) pGV series: The phytohormone genes are removed and replaced with a portion of the pBR322 vector sequence. The Ti plasmid's nopaline synthase gene and left and right border sequence are both conserved.

2. Intermediate vectors

These are small plasmids (*E. coli* vectors) based on pBR322 that have a T-DNA region. Intermediate vectors can multiply 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 co-integrated T-DNA structure[38].

3. Helper vectors

These are small plasmids maintained in E. coli that contain transfer (tra) and mobilization (mob) genes that allow the conjugation-deficient intermediate vectors to be transferred into Agrobacterium[38].

With the help of these three modified Agrobacterium Ti plasmid, two main plant vectors can be formed: a) Co integrative vectors b) Binary vectors.

a. Co-integrative vectors:

Vectors that recombine via DNA homology into a resident Ti plasmid are called Integrative or cointegrate vectors. A co-integrated vector is produced by integrating the modified E. coli plasmid into a disarmed pTi. A co-integration vector is produced by homologous recombination between two plasmids[6].

For example; one plasmid is PTiC58 (nopaline type Ti plasmid) which was disarmed by replacing its oncogenes with E. coli plasmid pBR322 sequences, co-integrative vector produced so was named as pGV3850. This pGV3850 disarmed ti plasmid contains all the regions present on normal Ti plasmid such as vir region, right and left border sequence except T-DNA region.

Another plasmid is pBR322 E-coli plasmid which is suitably modified to produce an intermediate vector(iv). pBR322 (IV) Must Contain Ori from E. coli, pBR322 sequence present in T-region of disarmed pTi, T-DNA from pTi, Appropriate selectable marker, (e.g.Neo gene for selection of plant cells containing recombinant T-DNA and Kanamycine resistance for the selection of co integrate vector in Agrobacterium.) except Ori for Agrobacterium.

This intermediate plasmid is a type of modified E-coli plasmid and therefore it is maintained in E-coli cells and disarmed pTi is maintained in agrobacterium cells. Transfer of recombinant IV from E. coli into Agrobacterium is usually occurred by conjugation process. Since pBR322(iv) is non-conjugative, an E. coli strain containing conjugation proficient plasmid called Helper plasmid (pRK2013) can be used. Through the helper plasmid, this pBR322 (iv) transformed from the E-coli to the agrobacterium cell.

The homologous recombination occurs between the two pBR322 sequences of both the plasmid and forms a new plasmid which is known as a co-integrative vector. The vir genes, the left and right T-DNA borders, an exogenous DNA sequence between the two T-DNA borders, and plant and bacterial selectable markers are often present in a co-integrated vector constructed in vitro.

Co-integrated vectors, also known as hybrid Ti plasmids, were among the earliest forms of modified and engineered Ti plasmids developed for Agrobacterium-mediated transformation, but they are no longer routinely utilized.

b. Binary vectors:

A binary vector consists of a pair of plasmids together that induce the transfer of transgene or DNA inserts into plant cells. Out of this pair of plasmid, one plasmid is a helper plasmid and another plasmid is a disarmed Ti plasmid[6].

One of the examples of disarmed Ti plasmid is BIN 19. BIN 19 contains Left border and right border sequence, DNA insert, Two selectively markers: kanamycin and neomycin resistance genes, Ori site for both E-coli and agrobacterium. This disarmed Ti plasmid is also called mini Ti/micro Ti plasmid. It is one type of modified E-coli plasmid, therefore it can be maintained in E-coli cells.

The example of helper Ti plasmid is PAL44O4. This plasmid only contains the vir gene in it. This helper plasmid with the disarmed plasmid catalyzes the insertion of the transgene from the agrobacterium into the plants. This helper plasmid is cloned in agrobacterium therefore, it is maintained in agrobacterium cells.

Now for the process, BIN 19 is transformed from the E-coli to agrobacterium by conjugational transfer. As BIN 19 plasmid contains ori site for both *E. coli* and agrobacterium and PAL44O4 plasmid contains ori site only for agrobacterium, both can be maintained in agrobacterium. Now, the presence of two vectors into a single Agrobacterium cell which can be transformed to plant cells is known as a binary vector. MINI Ti has kanr genes for the selection of Agrobacterium cells containing Bin 19 and Neo gene for the Selection of transformed plant cells. A binary vector avoids the transfer of unnecessary sequences into plant cells which occurs in the case of co-integrate vectors.

Agrobacterium-mediated genetic transformation of tobacco nodal segments *in vitro*

Generally, Agrobacterium-mediated genetic transformation is a natural gene transformation method but in the last few years, several in-vitro (artificial) methods have been developed for gene transformation via *A. tumefaciens*. For greater efficiency and steady transformation, scientists have included numerous alterations in the transformation process, such as the addition of acetocyronigen and adjustments in the co-cultivation times[39,40] . Plant transformation can use a variety of tissues and organs as explants, including leaves, callus, roots, cotyledons, and shoots[41]. The efficiency of plant transformation is determined by a mix of parameters such as the method utilized, the species, and Agrobacterium strain specificity[42].

Arabidopsis thaliana and Nicotiana tabacum plants have been used as plant models for many years as they have useful characteristics such as a large number of progeny and short generation time[43,44] which helps in the development of improved plant transformation methods for studying genes of interest[45]. Here, we have mentioned differently *in vitro* methods for infection, regeneration and genetic transformation of nodal segments of *N. tabacum* via *A. tumefaciens*.

Biological Material

The researchers employed nodal segments and foliar discs from wild-type tobacco (*N. tabacum*) seedlings that were pre-germinated *in vitro* in a controlled environment[46]. They used competent cells from two bacterial strains for the transformation process: A. tumefaciens for the indirect transformation of the plant and *Escherichia coli* for the assembly of the expression vector. In different mediums, *E. coli* and *A. tumefaciens* were cultivated[47].

Methods

Different three methods have been used for the transformation process in N. tabacum via A. tumefaciens which are the syringe method (SY), foliar disk method (FD) and nodal segment method (NS).

All the explants (nodel segment and foliar disk) used in this process have been sterilized and disinfected under the laminar airflow chamber by sterile water and 70% ethanol[46].

1. **Syringe method (SY):** Small punctures have been made in the meristematic region of the nodel segment with the use of a needle. A small amount of *A. tumefaciens* solution with plasmid was injected in that perforation with a syringe. Then this explant was transferred to the flask containing MS (murashige and Skoog) medium under controlled growth conditions. Then apply a specific amount of acetosyringone at the puncture region and allow it for cultivation[46].

2. **Foliar disk method (FD):** Take sterile tobacco leaves disks and dip them in the tube having a mixture of polypropylene, A. tumefaciens cell suspension and acetosyringone for at least 30 minutes co-cultivation period. Then transfer these disks in a flask containing MS medium[46].

3. **Nodal segment method (NS):** Make small wounds on the surface of nodel segments using a sterile scalpel then kept it in the tube having a mixture of polypropylene, *A. tumefaciens* cell suspension and acetosyringone for 30 minutes co-cultivation period. Then transfer it in a flask containing MS medium[46].

Flasks with the infected explants are transferred to a growth chamber under controlled growth conditions. After 72 hours, the explants are transferred to the new flask with MS medium containing cefotaxime antibiotic for the elimination of *A. tumefaciens* bacteria and kanamycin for transformant selection. Explants are transferred every 20 days to new flasks of MS medium having decreasing concentrations of cefotaxime antibiotic for complete removal of *A. tumefaciens*[46].

DNA can be extracted from leaves after plant regeneration and transformants can be confirmed by the PCR method.

The syringe and nodal segments methods show higher regeneration efficiency as A. tumefaciens solution was applied directly to meristematic tissue. And meristematic tissue generally had high differentiation efficiency which allows for faster plant regeneration[46]. In the leaf disk method, morphogenesis of leaves was interrupted, no plant regeneration was achieved. This problem of the leaf disk method can be overcome by using phytohormones such as BAP (6-benzyl amino purine) and 2,4-D (dichlorophenoxyacetic acid) for the induction of the regeneration process[46].

Scientists found from this experiment that syringe methods provided faster plant regeneration with higher transformation efficiency than any other method since they did not require time for bacterium inoculation and no co-cultivation of explants with *A. tumefaciens*[46]. The syringe method was also faster than other reported methods that require from one hour[39] to overnight growth[48] for the co-cultivation period when compared.

Utilizing explant's negative atmospheric pressure to induce transformation efficiency via *A. tumefaciens*

Scientist Beyaz *et al*.[49] has conducted this experiment on the flax plant (*Linum usitatissimum* L.). The primary goal of this experiment is to improve transformation efficiency by increasing the osmotic pressure of flax plant tissue. The flax seeds plant was used as an explant in this study. For transformation, an A. tumefaciens strain containing a plasmid containing the neomycin phosphotransferase II (npt-II) gene was used. Then this A. tumefaciens strain has grown in a liquid Nutrient Broth (NB) medium containing rifampicin and kanamycin at 28°C in a rotary shaker and used for the transformation process as a bacterial solution. Two different cultures of the same plant have been cultured in which One was through conventional transformation method in which hypocotyls of the plant were directly cultured on co-cultivation medium after inoculation with a bacterial solution and another one was prepared from the seeds of the flax plant. Seeds were sterilized and cultured in murashige and Skoog (MS)[50] medium for 7 days for germination and seedling establishment. Flex seedlings with cotyledon leaves but no root system were dried in laminar flow and infected with a bacterial solution at 7 days old. In both the transformation methods, hypocotyl segments were cultured on a co-cultivation medium for 2 days after inoculation with a bacterial solution. Then, both these explants were transferred to a regeneration medium containing a mixture of BAP, NAA, kanamycin, and augmentin and cultured for 4 weeks in a culture room at controlled growth conditions. Shoots were transferred to a rooting medium containing indole-butyric acid (IBA) and kanamycin in Magenta vessels to culture for 3 weeks at room temperature. After root formation, plantlets were transferred to pots and put it in the growth room for 3 weeks to get transgenic plants. PCR was used to check the presence of the neomycin phosphotransferase II (npt-II) gene in transformants.

Lowest transformation in the first method in which hypocotyls were directly cultured on co-cultivation medium after bacterial inoculation and highest transformation in the second method in which he kept 7-days old seedlings for germination. It was shown that by placing cotyledon leaves without root systems developed from 7-day old seedlings in laminar airflow, seedlings were able to ingest bacterial solution fast toward inner cells due to increased osmotic pressure, hence increasing transformation efficiency.

This work demonstrates that plant transformation efficiency can be enhanced by allowing seedlings to rapidly absorb bacterial solution into inner cells via increased osmotic pressure of explants.

The effect of gamma radiation on *A. tumefaciens*-mediated transformation

Generally, gamma radiation is inhibitory[52] for most of the lab process as well as harmful to work with. It gives rise to biochemical, morphological, cytological and physiological changes in cells by producing free radicals in it[51]. But it was noted that low doses of gamma rays are stimulatory. And they are used to increase germination, enzyme activity, cell proliferation, crop yields, stress resistance and cell growth[53-56].

The effects of gamma radiation from radioactive cobalt (60Co) rays on A. tumefaciens-mediated gene transfer in flaxseed are investigated in this experiment. Flax seeds were subjected to various gamma dosages. Following that, the flax seeds' surfaces were sterilized using commercial bleach containing 5% sodium hypochlorite. This mixture was strung continuously for greater surface sterilization before being washed three times with sterile distilled water at a particular temperature. After sterilizing the seeds, they were transferred to a pink jar containing MS medium for germination. After seven days of seed germination, hypocotyls were used for regeneration. For this experiment, A. tumefaciens strain harboring plasmid containing kanamycin resistance npt-II gene has been used for transformation studies. Hypocotyl were inoculated in the solution containing sterile water and bacterial solution. These inoculated hypocotyls were then cultured on MS medium containing BAP and NAA for co-cultivation at room temperature in the culture room. Then this explant with the different range of gamma doses were transferred to the selection medium containing BAP, NAA, kanamycin, duocid and cultured it for four weeks. Shoots of these cultured hypocotyls were transferred to the rooting medium containing indole-butyric acid (IBA) and kanamycin in Magenta vessels for 3 weeks at room temperature for the root formation. Then this newly developed, plantlets were transferred to the pot for the development for 3 weeks. Transformed plants having npt-II gene then can be determined by PCR analysis[46].

100% transformation efficiency has been recoreded at low gamma radiation, there is maximum transformation takes place in plants. And this lowest transformation efficiency is far better than no gamma radiation[46] .

CONCLUSION

Agrobacterium-mediated genetic transformation is now a widely used method in genetic engineering. The Agrobacterium-mediated transformation techniques vary from plant species to plant species, and even within species, from cultivar to cultivar. As a result, optimizing Agrobacterium-mediated transformation techniques necessitates taking into account a number of criteria that can be determined in the effective transformation of a single species. Here, We provided brief information about the AMT mechanism, including the history of A. tumefaciens-causes crown gall disease and the general protocol of Agrobacterium-mediated transformation in plants. As a plant pathogen, A. tumefaciens naturally infects wound sites in dicotyledonous plants and causes crown gall disease, and this bacterium has been widely used for the introduction of foreign genes into plants to achieve a desired phenotype in a variety of crops, and subsequent regeneration of transgenic plants. For many years, the fundamental understanding underlying the molecular process of Agrobacterium-mediated plant transformation has been a hot issue. This mechanism involves some important events such as bacterial attachment to the plant cell, vir gene activation, T-DNA processing, nuclear targeting, and T-DNA integration. However, the role of the host cellular proteins involved in the transformation process is also very important and is still being researched extensively. Still, in most of the plant species, Gene transfer mediated by A. tumefaciens is extremely challenging. Because the plant's defense mechanism is activated when a pathogen assaults, the success of genetic transformation via A. tumefaciens is limited. That is why plant and bacterium modifications, as well as physical circumstances, have been used to boost bacterium virulence and transformation efficiency. A deeper understanding of all molecular steps in the process, as well as the plant proteins involved, could be used to improve Agrobacterium-mediated plant transformation in the future. Furthermore, understanding the elements that influence transformation efficiency is critical. A detailed understanding of the parameters limiting transformation efficiency will increase the spectrum of crop species that can be changed by A. tumefaciens, particularly for refractory species.

Conflict of Interest

None declared.

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