Agrobacterium-based vectors: a review

Reza Mohammadhassan1*, Bahareh Kashefi2 and Kobra Shabanzadeh Delcheh1

1. M.Sc. student, Department of Agricultural Biotechnology, College of Agriculture, Damghan Branch, Islamic Azad University, Damghan, Iran
2. Assistant professor, Department of Horticulture Science, College of Agriculture, Damghan Branch, Islamic Azad University, Damghan, Iran

Corresponding author: Reza Mohammadhassan

ABSTRACT: Using a vector is needed for transferring a foreign DNA into a host cell. The vectors are mostly DNA molecules and sometimes have the essential factors for expressing gene. The suitable vector is selected based on the type of host cell and objective of experiment. Gene transferring improves by genetic engineering. Development of cloning vectors for higher plants was in the 1980s. The use of Agrobacterium is the most effective method of gene transferring to plants. In the article, we try to introduce Agrobacterium-mediated vector and virulence mechanism.

Keywords: Agrobacterium tumefaciens, Ti-plasmid, Binary vector, Agrobacterium rhizogenes, Ri-plasmid

INTRODUCTION

Modern biology has been changed in 1970s In these years a new technology was developed. This method was known as genetic engineering. Transfer a desirable gene from one organism to another organism, integration into the genome and stable gene expression in the acceptor is called "transformation". The transferred gene and the organism which obtained after a gene transfer successfully, are called Transgene and transgenic, respectively. Transgenic plants are carrying stable genes from external sources (Brown, 2010). For transferring a foreign DNA into a host cell, using a vector is required in most cases. The vectors are mainly DNA molecules which transfer gene into the host cell (bacteria, plants, animals) and have the necessary elements for gene expression in some cases. The desired vector is selected based on the type of host cell and objective of experiment. Plant gene transfer technology is essentially molecular plant genetics technique and product improvement by genetic engineering (Thole, 2007). Cloning vectors for higher plants were developed in the 1980s and their use has led to the genetically modified (GM) crops that are in the headlines today. Three types of vector system have been used with varying degrees of success with higher plants; such as vectors based on naturally occurring plasmids of Agrobacterium, direct gene transfer using various types of plasmid DNA and vectors based on plant viruses (Brown, 2010). The most effective method of gene transferring to plants is the use of Agrobacterium (Henry, 1997). In this paper, we try to introduce Agrobacterium-mediated vector, and discuss how their application in the genetic engineering.

Agrobacterium Tumefaciens

Agrobacterium tumefaciens is a Gram-negative soil bacterium and plant pathogen causing crown gall disease (Smith, 1907). It is pathogenic to a range of dicot plant species, causing the formation of crown galls or tumors at or close to infection sites (Hellens, 2000). Agrobacterium-plant interaction was one of the first model systems in which the molecular mechanism for plant pathogenicity has been elucidated in details (Chilton, 1977). About 20 kb segment of DNA (T-DNA) in a tumor-inducing plasmid (ca. 200 kb Ti plasmid) is transferred from the bacterium to the host plan genome by a molecular machinery closely resimilar to a bacterial conjugal transfer (Sheng, 1996). The disease phenotype is a manifestation of expression of bacterial T-DNA genes in plant cells that is over-production of two plant growth hormones, cytokinin and auxin (Murai, 2013). The proliferated tissue in the tumor provides the
bacterium with unusual amino acids (opines), which are an important carbon and nitrogen source, at the expense of the host plant. Genes required to establish a tumor and to bring about opine biosynthesis are transferred from *Agrobacterium* and hence this bacterium has been called Nature’s genetic engine (Hellens, 2000).

**Ti-plasmid**

Most of the machinery necessary for this T-DNA transfer resides on a tumor-inducing (Ti) plasmid. This Ti plasmid includes the T-DNA itself, delimited by 25 bp imperfect repeats [known as the right and left borders (RB and LB, respectively)] that define the boundaries of the T-DNA and ~35 virulence (vir) genes, clustered together into a vir region. The combined action of the vir genes achieves the delivery of the T-DNA to the nucleus of the host plant cell (Gelvin, 2003). In addition; some Ti-plasmids have a conjugative gene (Fullner, 1996).

**T-DNA**

*Agrobacterium* transfers T-DNA, which makes up a small (approximately 5%–10%) region of a resident Ti-plasmid (Lee, 2008). T-DNA contains the genes for inducing tumor formation and opines biosynthesis, and these genes, even though they are bacterial in origin, have evolved to function only in plant cells (Hellens, 2000). Also T-DNA encode the enzymes responsible for making Nopaline and Octopine (two type of Opines). These enzymes are Nopaline synthases (Nos) and Octopine synthases (Ocs), respectively. Based on type of Opine, Ti-plasmid is named (Fullner, 1996).

**Virulence genes**

The vir region consists of approximately 10 operons that serve four major functions (Lee, 2008). Sensing plant phenolic compounds and transducing this signal to induce expression of vir genes (virA and virG). VirA and VirG compose a two-component system that responds to particular phenolic compounds produced by wounded plant cells (Stachel, 1986). Because wounding is important for efficient plant transformation, *Agrobacterium* can sense a wounded potential host by perceiving these phenolic compounds. Activation of VirA by these phenolic inducers initiates a phospho-relay, ultimately resulting in phosphorylation and activation of the VirG protein (Winans, 1991). Activated VirG binds to the vir box sequences preceding each vir gene operon, allowing increased expression of each of these operons (Pazour, 1990). In addition to induction of the vir genes by phenolics, many sugars serve as co-inducers. These sugars are perceived by a protein, ChvE, encoded by a gene on the *Agrobacterium* chromosome. In the presence of these sugars, vir genes are more fully induced at lower phenolic concentrations (Peng, 1998). Processing T-DNA from the parental Ti-plasmid (virD1 and virD2). Together, VirD1 (a helicase) and VirD2 (an endonuclease) bind to and nick DNA at 25-bp directly repeated T-DNA border repeat sequences (Jayaswal, 1987). The VirD2 protein covalently links to the 5′ end of the processed single-strand DNA (the T-strand) and leads it out of the bacterium, into the plant cell, and to the plant nucleus (Howard, 1992). Secreting T-DNA and Vir proteins from the bacterium via a type IV secretion system (virB operon and virD4). The *Agrobacterium* virB operon contains 11 genes, most of which form a pore through the bacterial membrane for the transfer of Vir proteins (Christie, 2005). Currently, we know of five such proteins that are secreted through this apparatus: VirD2 (unattached or attached to the T-strand), VirD5, VirE2, VirE3, and VirF (Vergunst, 2005). VirD4 acts as a coupling factor to link VirD2-T-strand to the type IV secretion apparatus (Christie, 2005). Participating in events within the host cell involving T-DNA cytoplasmic trafficking, nuclear targeting, and integration into the host genome (virD2, virD5, virE2, virE3, and virF). VirD2 and VirE2 may play roles in targeting the T-strand to the nucleus (Zupan, 1996). In addition VirE2 likely protects T-strands from nucleolytic degradation in the plant cell (Rossi, 1996). VirF may play a role in stripping proteins off the T-strand prior to T-DNA integration (Tzfira, 2004). Although vir genes were first defined genetically because of their importance in virulence (Leemans, 1981), no gene within T-DNA is essential for T-DNA transfer. The ability to delete wild-type oncogenes and opine synthase genes from within T-DNA and replace them with genes encoding selectable markers and other *goi* helped initiate the field of plant genetic engineering (Bevan, 1983).
Table 1. Some disarmed Agrobacterium strain defined by Agrobacterium chromosomal background and the Ti plasmid they harbor (Hellens, 2000)

<table>
<thead>
<tr>
<th>Agrobacterium Strain</th>
<th>Chromosomal Background</th>
<th>Marker gene</th>
<th>Ti-plasmid plasmid</th>
<th>Marker gene</th>
<th>Opine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404</td>
<td>TiAch5</td>
<td>rif</td>
<td>pAL4404</td>
<td>spec &amp; strep</td>
<td>Octopine</td>
</tr>
<tr>
<td>GV2260</td>
<td>C58</td>
<td>rif</td>
<td>pGV2260</td>
<td></td>
<td>Octopine</td>
</tr>
<tr>
<td>GV3850</td>
<td>C58</td>
<td>rif</td>
<td>pGV3850</td>
<td>Carb</td>
<td>Nopaline</td>
</tr>
<tr>
<td>GV3101::pMP90</td>
<td>C58</td>
<td>rif</td>
<td>pMP90</td>
<td>Gent</td>
<td>Nopaline</td>
</tr>
<tr>
<td>GV3101::pMP90RK</td>
<td>C58</td>
<td>rif</td>
<td>pMP90RK</td>
<td>gent &amp; kan</td>
<td>Nopaline</td>
</tr>
<tr>
<td>EH101</td>
<td>C58</td>
<td>rif</td>
<td>pEH101</td>
<td>kan</td>
<td>Nopaline</td>
</tr>
</tbody>
</table>

**Using in Biotechnology**

It was realized very quickly that the Ti plasmid could be used to transport new genes into plant cells (Brown, 2010). This natural DNA transfer system has been exploited to introduce genes of agronomic interest into plants which resulted in the production of genetically modified crops by plant biotechnology companies (Murai, 2013). However, regeneration of a transformed plant can occur only if the Ti vector has been disarmed so that the transformed cells do not display cancerous properties (Brown, 2010). Agrobacterium-mediated transformation systems take advantage of this natural plant transformation mechanism. Removal of all the genes within the T-DNA does not impede the ability of Agrobacterium to transfer this DNA but does prevent the formation of tumors. Ti plasmids and their host Agrobacterium strains that are no longer oncogenic are termed 'disarmed' (Hellens, 2000).

In practice using of Ti-plasmid has proved a tricky proposition, mainly because the large size of the Ti plasmid makes manipulation of the molecule very difficult. The main problem is, of course, that a unique restriction site is impossibility with a plasmid 200 kb in size. Novel strategies have to be developed for inserting new DNA into the plasmid. Two are in general use (Brown, 2010). Initial approaches of gene transfer were to introduce a target gene into the T-DNA region of Ti plasmid after either a single-(co- integration) or double-homologous recombination between an intermediate vector (pRK290) and Ti plasmid (Zambryski, 1983). A binary plant vector strategy was designed to separate the T-DNA region in a small plasmid from the virulence genes in a virulent T-DNA-less Ti plasmid (Hoekema, 1983).

**Co-integrating method**

This method uses an entirely new plasmid, based on an E. coli vector, but carrying a small portion of the T-DNA. The homology between the new molecule and the Ti plasmid means that if both are present in the same A.tumefaciens cell, recombination can integrate the E. coli plasmid into the T-DNA region. The gene to be cloned is therefore inserted into a unique restriction site on the small E. coli plasmid, introduced into A. tumefaciens cells carrying a Ti plasmid, and the natural recombination process left to integrate the new gene into the T-DNA. Infection of the plant leads to insertion of the new gene, along with the rest of the T-DNA, into the plant chromosomes (Brown, 2010).

**Binary vector**

It is based on the observation that the T-DNA does not need to be physically attached to the rest of the Ti plasmid. A two-plasmid system, with the T-DNA on a relatively small molecule, and the rest of the plasmid in normal form, is just as effective at transforming plant cells. In fact, some strains of A. tumefaciens, and related agrobacteria, have natural binary plasmid systems. The T-DNA plasmid is small enough to have a unique restriction site and to be manipulated using standard techniques (Brown, 2010). The small plant vectors with the T-DNA region have been simply now called binary Ti vectors (Hellens, 2000).

**Composition**

Using the binary vector compared to Co-integrate vector has many advantages; such as:

1- The binary vector do not need for natural recombination in vivo, whereas co-integrating vector need it for permanent retention. Because Ti-plasmid has just a replication origin in co-integrating method. But both plasmids of binary system have a replication origin separately.

2- The rate of transformation for binary vectors is 2-3 days. But this rate for co-integrating vectors is 4-7 days.

3- Function of binary vectors is more natural. So they are more efficient than co-integrating vectors. In addition to natural recombination reduces efficiency of co-integrating method.

Today, virtually co-integrating vectors are not applied. Higher performance and ease of manipulation cause excellence and over application of binary vectors in genetic engineering of plants (Donson, 1991).

**Binary vector T-DNA Components**
A binary vector consists of T-DNA and the vector backbone. T-DNA is the segment delimited by the border sequences, the right border (RB) and the left border (LB), and may contain multiple cloning sites, a selectable marker gene for plants, a reporter gene, and other genes of interest. The vector backbone carries plasmid replication functions for E. coli and A. tumefaciens, selectable marker genes for the bacteria, optionally a function for plasmid mobilization between the bacteria and other accessory components (Komori, 2007).

**Borders**

The RB and the LB are imperfect, direct repeats of 25 bases and said to be the only essential cis-elements for T-DNA transfer (Yadav, 1982). The RB and the LB are integrated in binary vectors as DNA fragments cloned from well-known Ti plasmids, either octopine or nopaline type. Because factors that enhance T-DNA transfer have been identified near the borders (Wang, 1987), a few hundred bases of natural sequences adjacent to the T-DNA are retained by popular vectors (Komari, 1996).

**Selectable marker gene**

Choice of selectable marker genes is a key factor in plant transformation. Genes to give resistance to antibiotics or herbicides, such as kanamycin, hygromycin, phosphinothricin, and glyphosate, are very popular (Komari, 2007). Kanamycin resistance has been most frequently employed in the transformation of many dicotyledonous plants. Hygromycin resistance is the most effective in rice (Oryza sativa) transformation (Hiei, 1994), whereas phosphinothricin resistance is the most effective in maize (Zea mays; Ishida, 1996). If the development of herbicide-resistant plants is aimed at, a trait gene could also be a selectable marker gene. Because of concerns over antibiotic resistance genes in commercial transformants, genes to add metabolic capabilities have been drawing considerable attention. For example, plant cells expressing a phospho-Man isomerase can grow on media with Man as the sole carbon source. Such markers are referred to as positive selection markers (Joersbo, 1998). Although more than 20 selectable marker genes have been reported in the transformation of higher plants (Komari, 2006) to date, many of them were tested only in a limited number of plant species on a limited scale. Therefore, further studies of marker genes may contribute to improvement of the transformation of certain plant species (Komari, 2007). Genes that can confer resistance to kanamycin, gentamycin, tetracycline, chloramphenicol, spectinomycin, and hygromycin are popular markers for bacterial selection for both E. coli and A. tumefaciens. Care must be exercised as some bacterial strains without vector plasmids have certain intrinsic antibiotic resistance. Some selectable markers for plants, such as Nos-nptII and 35S-hpt, give fair levels of resistance to both E. coli and A. tumefaciens (Komari, 2006).

**Reporter gene**

β-Glucuronidase (Gus) (Jefferson, 1987), green fluorescent protein (GFP) (Pang, 1996) and luciferase (Luc) (Ow, 1986) are the most popular reporter genes. Background activities in the assays of these enzymes are generally very low in higher plants. Reporter genes can be linked to regulatory sequences and used to study functionality of these sequences (Komari, 2006). Because Gus and GFP are highly stable proteins in plant cells (Ruijter, 2003), the activity of these proteins may not immediately reflect small or quick changes in the level of the mRNA for these proteins in plant cells (Komari, 2006). If this is the case, Luc, whose half-life in plant cells is much shorter than those of Gus and GFP (Ruijter, 2003), may be a good choice. A reporter gene that has an intron in the coding sequence, such as the intron-Gus gene (Ohta, 1990), is very useful because this gene is not expressed in A. tumefaciens. In addition, especially in monocotyledons, introns can enhance expression for some genes (Tanaka, 1990). Introns placed close to the N-terminal in the coding sequence and in the 5' untranslated region of a gene may be equally effective (Simpson, 1996).

**Promoter**

Selectable markers need to be expressed in calli, in cells from those plants that are being regenerated, or germinating embryos to facilitate plant transformation. Therefore, promoters for constitutive expression are preferred (Komari, 2006). Promoters used mainly for dicotyledonous plants include the 35S promoter from cauliflower mosaic virus (Odell, 1985) and promoters derived from Ti plasmids, such as nopaline synthase (Nos) (Depicker, 1982), octopine synthase (Ocs), mannopine synthase (Mas), ene 1, gene 2, and gene 7 (Barker, 1983). Popular promoters for monocotyledonous plants include the 35S promoter and the promoters from the ubiquitin (Ubi) gene of maize (Christensen, 1992) and the actin (Act) gene of rice (Zhang, 1991). The choice of promoters that drive the selectable marker genes affects the efficiency of transformation. For example, the Ubi promoter gave a frequency of transformation much higher than that of the 35S promoter in cereals (Ishida, 2004). It is convenient to have a reporter gene expressed in various tissues and organs, and so the promoter is often chosen from the same group of promoters.
that may be used for selectable marker genes, which include 35S, Ubi, Act, Nos, and other T-DNA promoters. In some of the vectors, the promoter for the selectable marker and the reporter is the same, but, generally speaking, avoidance of duplication of the same components is recommended (Komari, 2006).

Figure 1. Typical structure of a binary vector. Key components and their major options are displayed (Komari, 2007).

**Agrobacterium Rhizogenes**

*Agrobacterium rhizogenes* is a gram negative soil bacterium. It incites hairy root disease of many dicotyledonous plants. The ability of *A. rhizogenes* to incite hairy root disease is determined by a virulence plasmid, similar to that found in *Agrobacterium tumefaciens* which causes Crown gall tumors of plants. The virulence plasmid of *A. rhizogenes* is known as the Ri-plasmid to distinguish it from the tumor-inducing (Ti) plasmid (Sinkar, 1987).

**Ri-plasmid**

Ri and Ti plasmids are very similar, the main difference (Brown, 2010) including size, T-DNA, opines and a gene in virulence (vir) region (Christey, 2005). The Ri–plasmid contains a distinct segment (s) of DNA which is transferred to plant genome during infection. The transfer of the DNA (T-DNA) to the plant genome is mediated by another segment on the plasmid known as the virulence (vir) region. The T-DNA confers on the plant cells the ability to grow in the absence of exogenous plant hormones. Large plasmids were shown to be present in strains of *A. rhizogenes*. These strains are known to produce at least two classes of opines. One such class is represented by opines of agropine group, and the other class being the agrocinopine group. All strains of *A. rhizogenes* are known to produce agrocinopine and all or a few opines of the agropine group (Sinkar, 1987).

**T-DNA**

Two T-DNA regions have been identified in agropine Ri-plasmids. The two tDNAs are separated from each other by about 15 Kb of non-transferred DNA. The right T–DNA (TR) contains genes homologous to the T–DNA from Ti–plasmids. Most important among these are the genes homologous to the *tms1* and *tms2* of the Ti-plasmid. These genes are involved in auxin biosynthesis in *A. tumefaciens*. The left T-DNA (TL) of agropine Ri-plasmid A4b is about 20 Kb in length but, unlike the TR–DNA does not appear to be closely related to any other characterized Ti-plasmid. Limited homology has been reported to the T– DNA of nopaline type Ti-plasmids, presumably to the region involved in the synthesis of agrocinopine (Sinkar, 1987).

**Virulence gene**

The physical structure and hybridization data strongly suggest a functional similarity between vir regions of the Ri- and Ti-plasmids. One exception to this finding is the lack of hybridization to virE probe. Vir mutations in Ti-plasmid including virE can, however, be complemented by the virulence region of the Ri–plasmid (Sinkar, 1987). DNA transfer occurs without two essential proteins (VirE1 and VirE2) found *Agrobacterium tumefaciens*. GALLS gene replaces VirE2 in Ri-plasmid. Similarities between GALLS and VirE2 contain a nuclear localization signal; however they are not the same amino acid sequence and same type four secretion signal. Also differences between them include nucleotide sequences show no homology at the first; The second, only GALLS has a nucleoside triphosphate binding motif. The 3rd, GALLS has a molecular weight more than three times that of VirE2 and the last, there is no evidence of a chaperone for GALLS (Christey, 2005).
Using in Biotechnology

Over the years there has also been interest in developing plant cloning vectors based on the Ri plasmid of Agrobacterium rhizogenes (Brown, 2010). The Ri-plasmid shares extensive functional homology with the Ti-plasmid (Sinkar, 1987). The possibility of growing transformed roots at high density in liquid culture has been explored by biotechnologists as a potential means of obtaining large amounts of protein from genes cloned in plants (Brown, 2010).

REFERENCES


