A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*

**Frequent co-transformation of two distinct T-DNAs**

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**Summary**

Binary Ti plasmid vector systems consist of two plasmids in *Agrobacterium*, where one plasmid contains the DNA that can be transferred to plant cells and the other contains the virulence (*vir*) genes which are necessary for the DNA transfer but are not themselves stably transferred. We have constructed two non-oncogenic vectors (pARC4 and pARC8) based on the binary Ti plasmid system of *Agrobacterium tumefaciens* for plant transformation. Each vector contains the left and right termini sequences from pTiT37. These sequences, which determine the extent of DNA transferred to plant cells, flank unique restriction enzyme sites and a marker gene that functions in the plant (nopaline synthase in pARC4 or neomycin phosphotransferase in pARC8). After construction in vitro, the vectors can be conjugatively transferred from *E. coli* to any of several *Agrobacterium* strains containing *vir* genes. Using *A. rhizogenes* strain A4 containing the resident Ri plasmid plus a vector with the nopaline synthase marker, we found that up to 50% of the hairy roots resulting from the infection of alfalfa or tomato synthesized nopaline. Thus, vector DNA encoding an unselected marker was frequently co-transferred with Ri plasmid DNA to an alfalfa or a tomato cell. In contrast, the frequency of co-transfer to soybean cells was difficult to estimate because we encountered a high background of non-transformed roots using this species. Up to five copies of the vector DNA between the termini sequences were faithfully transferred and maintained in most cases suggesting that the termini sequences and the *vir* genes from the Ri and Ti plasmids are functionally equivalent.

**Introduction**

Because of their natural ability to transfer DNA to plant cells, *Agrobacterium tumefaciens* and its Ti plasmids have been used as vectors to introduce foreign DNA into plants (as reviewed recently 27, 34, 54). Since the production of auxins and cytokinins by transformed cells is often incompatible with normal plant regeneration, it is frequently desirable to 'disarm' the plasmids by removing the oncogenes responsible for the synthesis of these growth regulators and to introduce selectable or screenable markers in their place. The large size of the Ti plasmids makes it necessary to use intermediate vectors. One intermediate vector method is the 'co-integration' approach, whereby foreign DNA is inserted into a vector that cannot replicate in *Agrobacterium*, but can recombine with the Ti plasmid through a homologous portion of the vector, producing a co-integrate of the two plasmids (16, 21, 55). Another method is the binary vector approach whereby a foreign gene is inserted into a disarmed T-DNA which itself is joined to a broad host range replicon that can replicate in *Agrobacterium* (3, 6, 17, 24, 26, 29).

The mechanism of T-DNA transfer from the bacteria to the plant is not known in detail, but at a minimum, transfer requires termini sequences and *vir* genes from the Ti plasmid in addition to bacterial chromosomal genes. The termini sequences
are imperfect 25 basepair direct repeats found flanking the T-DNA, at least one of which is required for the transfer (9). The \textit{vir} genes are required for DNA transfer but are themselves not stably transferred to the plant (34). Although the \textit{vir} genes and the T-DNA are normally part of the same bacterial replicon, a binary system is possible, in which these functions are on separate replicons (17, 24, 26).

Since an oncogenic marker is not present in these disarmed vectors, other markers have to be used to identify genetically transformed plant cells. Some markers, such as enzymes which result in the production of opines, can be used to screen transformed tissue for the presence of opines (e.g., octopine, nopaline or agropine; 37). Other markers, such as enzymes which confer resistance to antibiotics, can be used to select transformed tissue which can grow in the presence of an antibiotic (e.g., kanamycin, chloramphenicol or methotrexate; 23).

\textit{Agrobacterium rhizogenes} (27) is considered a close relative of \textit{A. tumefaciens} because of its similar mechanism of plant transformation based on DNA transfer to plants, the similar function of the \textit{vir} genes, and the production of opines by transformed tissue. \textit{A. rhizogenes} frequently produces transformed, hairy roots so the endogenous plasmid has been called the Ri ('root-inducing') plasmid. Hairy roots from several species have been regenerated into plants which contain T-DNA from the Ri plasmid (10, 12, 43, 44). In cases where plants can be regenerated from roots, the combination of \textit{vir} genes from the Ri plasmid and a gene transfer vector derived from the T-DNA of \textit{A. tumefaciens} may be the system of choice for gene transfer.

We report here, the construction of binary vectors for use in \textit{A. tumefaciens} or \textit{A. rhizogenes} containing either, a selectable marker that confers kanamycin-resistance to transformed plant cells, or a marker that is easy to screen, nopaline synthase. Inoculation of several plant species with \textit{A. rhizogenes} containing a vector resulted in hairy roots. With alfalfa and tomato, we could demonstrate frequent co-transfer of vector DNA and Ri plasmid DNA. Southern analysis of the roots has shown that in most cases the DNA was transferred and integrated faithfully into the plant genome.

**Materials and methods**

The procedure for transformation of \textit{Escherichia coli} with plasmid DNAs is as described (1). Other manipulations of nucleic acids are essentially those described by Maniatis \textit{et al.} (32), unless otherwise indicated. Restriction enzymes and pUC8 were obtained from Bethesda Research Laboratories. The BglII linkers were from New England Biolabs. The bacterial strains and plasmids used for these experiments are listed in Table 1.

**Plasmid constructions**

The vector pARC4 was constructed as illustrated in Fig. 1A. The plasmid pBstEII 9, 14 (52) carries the 1.5 kb EcoRI fragment 29 derived from Ti plasmid pT1T37. This fragment contains the left terminus of the T-DNA region located approximately 50 basepairs from the right end of the fragment (51, 53). The plasmid pT37H23, a generous gift from Scott Stachel, carries the 3.2 kb HindIII fragment 23 from Ti plasmid pT1T37 (18). From left to right, this fragment contains the 5' portion of the DNA encoding transcript 6b, the entire nopaline synthase or NOS gene, the right terminus sequence and a portion of the Ti plasmid which is not transferred to plant cells.

The chimeric gene 'NOS/NPT' was constructed by placing the coding region for the neomycin phosphotransferase II gene (NPT) from the bacterial transposon Tn5 under control of the transcriptional regulatory signals of the nopaline synthase gene (NOS). The 'NOS/NPT' gene is part of the plasmid pNEO105 whose structure is shown schematically in the bottom panel of Fig. 2. The NOS gene fragment was derived from the plasmid pT37H23 (18). Based on the numbering convention of Depicker \textit{et al.} (18), pNEO105 contains the nopaline synthase promoter (from the BclI site at position -265 to position +30) and polyadenylation site (from the SphI site at position +1136 to the HindIII site at position +1972). The NPT fragment was derived from the plasmid pNEO (P-L Biochemicals) which contains the neomycin phosphotransferase II gene from Tn5 cloned into pBR322. Based on the numbering convention of Beck \textit{et al.} (4), pNEO105 contains a portion of the NPT gene (from position 1543 to the SmaI site at