T-DNA Binary Vectors and Systems

Lan-Ying Lee and Stanton B. Gelvin*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907–1392

For more than two decades, scientists have used *Agrobacterium*-mediated genetic transformation to generate transgenic plants. Initial technologies to introduce genes of interest (goi) into *Agrobacterium* involved complex microbial genetic methodologies that inserted these goi into the transfer DNA (T-DNA) region of large tumor-inducing plasmids (Ti-plasmids). However, scientists eventually learned that T-DNA transfer could still be effected if the T-DNA region and the virulence (*vir*) genes required for T-DNA processing and transfer were split into two replicons. This binary system permitted facile manipulation of *Agrobacterium* and opened up the field of plant genetic engineering to numerous laboratories. In this review, we recount the history of development of T-DNA binary vector systems, and we describe important components of these systems. Some of these considerations were previously described in a review by Hellens et al. (2000b).

Agrobacterium transfers T-DNA, which makes up a small (approximately 5%–10%) region of a resident Ti-plasmid or root-inducing plasmid (Ri-plasmid), to numerous species of plants (DeCleene and DeLey, 1976; Anderson and Moore, 1979), although the bacterium can be manipulated in the laboratory to transfer T-DNA to fungal (Bundock et al., 1995; Piers et al., 1996; de Groot et al., 1998; Abuodeh et al., 2000; Kelly and Kado, 2002; Li et al., 2007) and even animal cells (Kunik et al., 2001; Bulgakov et al., 2006). Transfer requires three major elements: (1) T-DNA border repeat sequences (25 bp) that flank the T-DNA in direct orientation and delineate the region that will be processed from the Ti/Ri-plasmid (Yadav et al., 1982); (2) *vir* genes located on the Ti/Ri-plasmid; and (3) various genes (chromosomal virulence [*chv*] and other genes) located on the bacterial chromosomes. These chromosomal genes generally are involved in bacterial exopolysaccharide synthesis, maturation, and secretion (e.g. Douglas et al., 1985; Cangelosi et al., 1987, 1989; Robertson et al., 1988; Matthysse, 1995; O'Connell and Handelsman, 1999). However, some chromosomal genes important for virulence likely mediate the bacterial response to the environment (Xu and Pan, 2000; Saenkham et al., 2007). Several recent reviews enumerate factors involved in and influencing *Agrobacterium*-mediated transformation (Gelvin, 2003; McCullen and Binns, 2006).

* Corresponding author; e-mail gelvin@bilbo.bio.purdue.edu.
www.plantphysiol.org/cgi/doi/10.1104/pp.107.113001

The *vir* region consists of approximately 10 operons (depending upon the Ti- or Ri-plasmid) that serve four major functions.

(1) 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 et al., 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 and Das, 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 et al., 1998).

(2) Processing T-DNA from the parental Ti- or Ri-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 et al., 1987; Wang et al., 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 (Ward and Barnes, 1988; Howard et al., 1992).

(3) 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 et al., 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 et al., 2000, 2005). VirD4 acts as a coupling factor to link VirD2-T-strand to the type IV secretion apparatus (Christie et al., 2005).

(4) 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 (Howard et al., 1992; Zupan et al., 1996). In addition,

VirE2 likely protects T-strands from nucleolytic degradation in the plant cell (Yusibov et al., 1994; Rossi et al., 1996). VirF may play a role in stripping proteins off the T-strand prior to T-DNA integration (Tzfira et al., 2004).

Although *vir* genes were first defined genetically because of their importance in virulence (Koekman et al., 1979; Garfinkel and Nester, 1980; Holsters et al., 1980; DeGreve et al., 1981; Leemans et al., 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 et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983).

DEVELOPMENT OF BINARY VECTOR SYSTEMS

Initial efforts to introduce goi into T-DNA for subsequent transfer to plants involved cumbersome genetic manipulations to recombine these genes into the T-DNA region of Ti-plasmids (co-integrate or exchange systems; Garfinkel et al., 1981; Zambryski et al., 1983; Fraley et al., 1985; Fig. 1A). This was because Ti/Ri-plasmids are very large, low copy number in *Agrobacterium*, difficult to isolate and manipulate in vitro, and do not replicate in *Escherichia coli*, the favored host for genetic manipulation. T-DNA regions from wild-type Ti-plasmids are generally large and do not contain unique restriction endonuclease sites suitable for cloning a goi. In addition, scientists wanted to eliminate oncogenes from T-DNA to regenerate normal plants. Opine synthase genes were also generally deemed superfluous in constructions designed to deliver goi to plants.

In 1983, two groups made a key conceptual breakthrough that would allow laboratories that did not specialize in microbial genetics to use *Agrobacterium* for gene transfer. Hoekema et al. (1983) and de Framond et al. (1983) determined that the *vir* and T-DNA regions of Ti-plasmids could be split onto two separate replicons. As long as both of these replicons are located within the same *Agrobacterium* cell, proteins encoded by *vir* genes could act upon T-DNA in trans to mediate its processing and export to the plant. Systems in which T-DNA and *vir* genes are located on separate replicons were eventually termed T-DNA binary systems (Fig. 1B). T-DNA is located on the binary vector (the non-T-DNA region of this vector containing origin[s] of replication that could function both in *E. coli* and in *Agrobacterium tumefaciens*, and antibiotic-resistance genes used to select for the presence of the binary vector in bacteria, became known as vector backbone sequences). The replicon containing the *vir* genes became known as the vir helper. Strains harboring this replicon and a T-DNA are considered disarmed if they do not contain oncogenes that could be transferred to a plant.

The utility of binary systems for ease of genetic manipulation soon became obvious. No longer were complex, cumbersome microbial genetic technologies necessary to introduce a goi into the T-region of a Ti-plasmid. Rather, the goi could easily be cloned into small T-DNA regions within binary vectors specially suited for this purpose. After characterization and verification of the construction in *E. coli*, the T-DNA binary vector could easily be mobilized (by bacterial conjugation or transformation) into an appropriate *Agrobacterium* strain containing a *vir* helper region.

Over the past 25 years, both T-DNA binary vectors and disarmed *Agrobacterium* strains harboring *vir* helper plasmids have become more sophisticated and suited for specialized purposes. Table I lists many commonly used T-DNA binary vectors (and vector series). Table II lists many commonly used disarmed *Agrobacterium vir* helper strains.

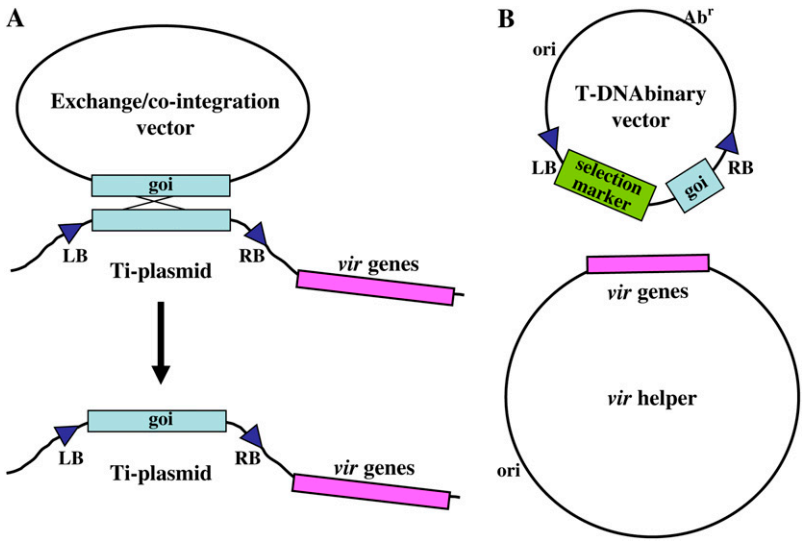

Figure 1. Schematic diagram of co-integration/exchange systems and T-DNA binary vector systems to introduce genes into plants using *Agrobacterium*-mediated genetic transformation. A, Co-integration/exchange systems. Genes of interest (goi) are exchanged into the T-DNA region of a Ti-plasmid (either oncogenic or disarmed) via homologous recombination. Following exchange, the exchange/co-integration vector can be cured (removed) from the *Agrobacterium* cell; B, T-DNA binary vector systems. Genes of interest are maintained within the T-DNA region of a binary vector. Vir proteins encoded by genes on a separate replicon (*vir* helper) mediate T-DNA processing from the binary vector and T-DNA transfer from the bacterium to the host cell. The selection marker is used to indicate successful plant transformation. ori, Origin of replication; Ab^r, antibiotic-resistance gene used to select for the presence of the T-DNA binary vector in *E. coli* (during the initial stages of gene cassette construction) or in *Agrobacterium*.

Table I. *Agrobacterium T-DNA binary vectors*

Vector Series Name	Vector ori/ Incompatibility Group	Important Features[a]	Gateway Compatable	Bacterial Selection Marker[b]	Plant Selection Marker[b]	Reference
pBIN	IncPα	mcs with blue/white selection	No	Kan	Kan	Bevan (1984)
pGA	IncPα	cos site ColE1 ori	No	Kan	Kan	An et al. (1985); An (1987)
SEV	IncPα	Reconstitutes a missing T-DNA border; not a binary vector	No	Kan	Kan/Nos	Fraley et al. (1985)
pEND4K	IncPα	cos site, mcs with blue/white selection	No	Kan/Tet	Kan	Klee et al. (1985)
pBI	IncPα	Promoterless *gusA* gene for promoter studies	No	Kan	Kan	Jefferson et al. (1987)
pCIB10	IncPα	Chimeric antibiotic-resistance gene	No	Kan	Chimeric Kan/Hyg	Rothstein et al. (1987)
pMRK63	pRi	pRi-based vector (borders from pRi)	No	Amp/Kan	Kan	Vilaine and Casse-Delbart (1987)
pGPTV	IncPα	Promoterless *gusA* gene for promoter studies	No	Kan	Kan/Hyg/Bar/ Bleo/Dhfr	Becker (1990)
pCGN1547	pRi + ColE1	ColE1 ori for high copy no. in *E. coli* mcs with blue/white selection	No	Gent	Kan	McBride and Summerfelt (1990)
pART	IncPα + ColE1	ColE1 ori for high copy no. in *E. coli* promoter/polyA expression cassette	No	Spec	Kan	Gleave (1992)
pGKB5	pRiA4	Promoterless *gusA* gene for promoter studies	No	Kan	Kan/Bar	Bouchez et al. (1993)
pMJD80 pMJD81	IncPα	Ω, untranslated leader	No	Kan	Kan	Day et al. (1994)
pPZP	pVS1	Small, stable, mcs with blue/white selection	No	Spec/Chl	Kan/Gent	Hajdukiewicz et al. (1994)
pBINPLUS	IncPα	Selectable marker near LB ColE1 ori	No	Kan	Kan	van Engelen et al. (1995)
pRT100 pRT-Ω/Not/Asc	IncPα	Rare-cutting sites (*Not*I, *Asc*I)	No	Kan	Kan/Hyg/ Bar/Dhfr	Uberlacker and Werr (1996)
BIBAC	pRi	T-DNA binary vector designed to transfer large DNA fragments	No	Kan	Hyg	Hamilton (1997)
pCB series	IncPα	Mini binary vectors small backbone, not self-mobilizable	No	Kan	Bar	Xiang et al. (1999)
pGreen	IncW	ColE1 ori mcs with blue/white selection	No	Kan	Kan/Hyg/ Sul/Bar	Hellens et al. (2000a)
pPZP-RCS2	pVS1	Multiple rare-cutting sites for cassette insertion. Uses pPZP200 as backbone	No	Spec	Kan/Gent	Goderis et al. (2002)
GATEWAY destination vector	pVS1	ColE1 ori. Uses pPZP200 as backbone	Yes	Spec	Kan/Hyg/Bar	Karimi et al. (2002)
pMDC	pVS1	Based on pCAMBIA (except pMDC7, from PER8). Facilitates protein tagging	Yes	Kan; Spec for pMDC7	Kan/Hyg/Bar	Curtis and Grossniklaus (2003)
pRCS2	pVS1	Contains rare-cutting sites	No	Spec	Kan/Hyg/Bar	Chung et al. (2005)
pRCS2-ocs	pVS1	Cloning of multiple genes	No	Spec	Kan/Hyg/Bar	Tzfira et al. (2005)
pEarleyGate	pVS1	Based on pCAMBIA. Facilitates protein tagging	Yes	Kan	Bar	Earley et al. (2006)
pGWTAC pMDC99	pRiA4	Multi-Round Gateway for cloning multiple genes	Yes	Kan	Hyg	Chen et al. (2006)
pORE	IncPα	Based on pCB301 ColE1 ori FRT sites. Promoterless *gusA* or *gfp* gene for promoter studies	No	Kan	Kan/Pat	Coutu et al. (2007)

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Vector Series Name	Vector ori/ Incompatibility Group	Important Features[a]	Gateway Compatable	Bacterial Selection Marker[b]	Plant Selection Marker[b]	Reference
pSITE	pVS1	Fluorescence protein fusion. Based on pRCS2	Yes	Spec	Kan	Chakrabarty et al. (2007)
pMSP	IncPα	Super-promoter to drive expression of goi	No	Kan	Kan/Hyg/Bar	Lee et al. (2007)
pCAMBIA	pVS1	Multiple vectors for cloning, expression, and tagging	No	Kan/Chl	Kan/Hyg/Bar	http://www.cambia.org/daisy/cambia/materials/vectors
pGD	PVS1	Derived from pCAMBIA1301. Multiple vectors for tagging proteins with DsRed2 or GFP	No	Kan	Hyg	Goodin et al. (2002)

[a]cos, Bacteriophage λ cohesive ends; mcs, multiple cloning site; ori, vegetative origin of replication; Ω, tobacco mosaic virus translational enhancer. [b]Amp, Ampicillin; Bar, resistance to phosphinothricin; Bleo, bleomycin; Chl, chloramphenicol; Dhfr, dihydrofolate reductase; Gent, gentamicin; Hyg, hygromycin; Kan, kanamycin, Nos, nopaline synthase; Pat, resistance to phosphinothricin; Spec, spectinomycin; Sul, sulfonylurea; Tet, tetracycline.

PROPERTIES OF BINARY VECTORS

T-DNA binary vectors generally contain a number of features important for their use in genetic engineering experiments. These include the following.

(1) T-DNA left and right border repeat sequences to define and delimit T-DNA. T-DNA border repeat sequences (T-DNA borders) contain 25 bp that are highly conserved in all Ti- and Ri-plasmids examined to date (Waters et al., 1991). Nicking by the VirD1/VirD2 endonuclease occurs between nucleotides 3 and 4 (Wang et al., 1987). Thus, within *Agrobacterium*, nucleotides 4 to 25 remain within the T-DNA at the left border (LB), whereas at the right border (RB) nucleotides 1 to 3 remain intact. However, within the plant, the T-strand is frequently chewed back, most likely by exonucleases. Because VirD2 is linked to and therefore protects the 5′ end of the T-strand, loss of nucleotides at this end is usually minimal (a few nucleotides at most). Loss of nucleotides from the unprotected 3′ end occurs more frequently and is generally more extensive; deletions up to several hundred nucleotides are not uncommon (Rossi et al., 1996). Early T-DNA binary vectors contained the plant antibiotic selection marker gene near the 5′ end of T-DNA (RB), and goi were placed near the 3′ end (LB; e.g. Bevan, 1984). However, extensive loss of DNA from the 3′ end, most likely the result of nucleolytic degradation, could result in antibiotic-resistant transgenic plants with deletions in the goi. This problem was ameliorated by placing the selection marker gene near the LB and the goi near the RB. Extensive deletion of the T-DNA from the 3′ end would result in removal of the selection marker and lack of recovery of these plants. Thus, deletion of the goi was generally abrogated. Sequences near RBs (so-called overdrive sequences) can increase transmission of T-DNA (Peralta et al., 1986). These sequences are frequently incorporated into T-DNA binary vector RB regions.

(2) A plant-active selectable marker gene (usually for antibiotic or herbicide resistance). The most commonly used selection systems employ aminoglycoside antibiotics such as kanamycin or hygromycin, herbicides such as phosphinothricin/gluphosinate, or herbicide formulations such as Basta or Bialophos. Other selection systems, such as phospho-mannose isomerase, employ metabolic markers (Todd and Tague,

Table II. *Frequently used disarmed Agrobacterium strains*

Strain Name	Chromosomal Background	Ti-Plasmid Derivation	Antibiotic Resistance[a]	Reference
AGL-0	C58	pTiBo542	rif	Lazo et al. (1991)
AGL-1	C58	pTiBo542	rif, carb	Lazo et al. (1991)
C58-Z707	C58	pTiC58	kan	Hepburn et al. (1985)
EHA101	C58	pTiBo542	rif, kan	Hood et al. (1986)
EHA105	C58	pTiBo542	rif	Hood et al. (1993)
GV3101::pMP90	C58	pTiC58	rif, gent	Koncz and Schell (1986)
LBA4404	Ach5	pTiAch5	rif	Ooms et al. (1982)
NT1(pKPSF2)	C58	pTiChry5	ery	Palanichelvam et al. (2000)

[a]carb, carbenicillin; ery, erythromycin; gent, gentamicin; kan, kanamycin; rif, rifampicin.

2001). Some plant species have low-level tolerance to kanamycin, and care should be taken to determine the minimum concentration of antibiotic that will completely kill nontransformed tissues. As mentioned above, early binary vectors had these markers placed near the T-DNA RB. However, because of the polarity of T-DNA transfer (RB to LB; Wang et al., 1984), recent vectors contain the selectable marker near the LB to assure transfer of the goi.

(3) Restriction endonuclease, rare-cutting, or homing endonuclease sites within T-DNA into which goi can be inserted. Early binary vectors, such as pBIN19, contained a few restriction endonuclease cloning sites in a *lacZ* α complementation fragment, permitting blue/white screening for the presence of the transgene insertion (Bevan, 1984). In many vectors, promoters and polyA addition signals flank these sites. More recently, binary vectors containing multiple rare-cutting restriction endonuclease or homing endonuclease sites have been developed (Chung et al., 2005; Tzfira et al., 2005). These vectors, derived from plasmids originally constructed by Goderis et al. (2002), are designed to accompany a series of satellite (pSAT) vectors. The pSAT vectors contain expression cassettes (promoter, multiple restriction endonuclease cloning sites, polyA addition signal) flanked by rare-cutting/homing endonuclease sites (Chung et al., 2005). Some of these vectors have incorporated into these expression cassettes tags to generate fluorescent fusion proteins for protein localization studies (Tzfira et al., 2005) or protein-protein interaction studies (Citovsky et al., 2006). Multiple expression cassettes from the pSAT vectors can be loaded into the cognate rare-cutting sites in the binary vectors, permitting simultaneous introduction of multiple genes into plants. Several recent binary vectors contain Gateway sites to facilitate insertion of genes or exchange of gene cassettes from other vectors. Additionally, several BAC binary vectors have been designed to clone large inserts of more than 100 kb (Hamilton, 1997; Liu et al., 1999, 2000).

(4) Origin(s) of replication to allow maintenance in *E. coli* and *Agrobacterium*. The incompatibility group of the plasmid, with function related to the specific origin of replication, can be important if several plasmids need to co-exist in the bacterium. As such, these plasmids must belong to different incompatibility groups. In some instances, origins of replication may function in both *Agrobacterium* and in *E. coli* (in which initial constructions are generally made). These broad host range replication origins include those from RK2 (incPα; e.g. pBIN19 and derivatives), pSa (incW; e.g. pUCD plasmid derivatives), and pVS1 (e.g. pPZP derivatives). Other origins of replication that function in *Agrobacterium*, such as those from Ri-plasmids (e.g. pCGN vectors), do not function in *E. coli*; thus, a ColE1 origin (such as the one used in pUC and pBluescript plasmids) is added to the vector. Different origins of replication replicate to different extents in *Agrobacterium*. The pSa origin replicates to two to four copies per cell (Lee and Gelvin, 2004), the RK2 (Veluthambi et al., 1987) and pVS1 (L.-Y. Lee, unpublished data) origins replicate to seven to 10 copies per cell, and the pRi origin replicates to 15 to 20 copies per cell (L.-Y. Lee, unpublished data).

(5) Antibiotic-resistance genes within the chromosome and within backbone sequences for selection of the binary vector in *E. coli* and *Agrobacterium*. Many commonly used *Agrobacterium* strains are resistant to rifampicin due to a chromosomal mutation (see Table II). In addition, commonly used *Agrobacterium* strains can be grown on Suc as the sole carbon source. Most commonly used *E. coli* K12 laboratory strains cannot use Suc as a carbon source. Thus, growth on minimal medium containing rifampicin and Suc generally will eliminate *E. coli* from *Agrobacterium* cultures, an especially useful selection following introduction of the binary vector into *Agrobacterium* by mating plasmids between *E. coli* and *Agrobacterium* (Ditta et al., 1980; Garfinkel et al., 1981).

Care must be taken in matching binary vectors with specific *vir* helper *Agrobacterium* strains. As listed in Table II, many of these strains already express genes for resistance to kanamycin, carbenicillin, erythromycin, or gentamicin. Thus, one cannot easily use binary vectors with the same selection marker in these strains. For example, many T-DNA binary vectors based upon pBIN19 utilize kanamycin-resistance as the bacterial selection marker. *A. tumefaciens* EHA101 is kanamycin resistant and cannot easily be used with these pBIN19 derivatives. However, one can use these binary vectors in the near-isogenic kanamycin-sensitive strain *A. tumefaciens* EHA105. In addition, some *Agrobacterium* strains are resistant to low levels of spectinomycin, an antibiotic that is used in conjunction with the pPZP plasmids and their derivatives. When using spectinomycin, the researcher should test various concentrations of the antibiotic with the *vir* helper strain lacking the binary vector to assure effective killing. Care must also be taken if a binary vector contains a tetracycline-resistance gene. *A. tumefaciens* C58 harbors a tetracycline-resistance determinant (Luo and Farrand, 1999) and is thus resistant to low levels of this antibiotic.

Although some *Agrobacterium* strains or binary vectors may harbor a β-lactamase gene that confers resistance to carbenicillin, it is still relatively easy to kill these bacteria following infection of plants. The β-lactam antibiotics Augmentin and Timentin contain, additionally, clavulanate, which will inhibit β-lactamases. Concentrations of Timentin ranging from 100 to 150 mg/L will completely eliminate growth of *Agrobacterium* C58-based strains harboring a β-lactamase gene (Cheng et al., 1998). *Agrobacterium* Ach5-based strains, such as LBA4404, do not express β-lactamase activity well, and thus can be killed by even lower concentrations of either carbenicillin or Timentin (Hooykaas, 1988).

ALTERNATIVE T-DNA BINARY SYSTEMS

Although T-DNA binary vector systems almost always consist of T-DNA and *vir* regions localized on

plasmids, it is not essential that they function this way. Replicons containing T-DNA or *vir* genes do not need to be plasmids. Indeed, several laboratories have shown that T-DNA can be integrated into an *Agrobacterium* chromosome and launched from this replicon (Hoekema et al., 1984; Miranda et al., 1992), and specialized vectors have been generated to facilitate integration of DNA into a specific neutral (i.e. not involved in virulence) region of the chromosome of *A. tumefaciens* C58 (Lee et al., 2001). Although launching T-DNA from the *Agrobacterium* chromosome can result in lower transformation frequencies, this process has the beneficial consequences of reducing integrated transgene copy number and almost completely eliminating integration of vector backbone sequences into the plant genome (Ye et al., 2007).

CONCLUSION

T-DNA binary systems have greatly simplified the generation of transgenic plants. No longer are complex, sophisticated microbial genetic regimens required to integrate goi into T-DNA regions located on large, cumbersome Ti- or Ri-plasmids. Along with companion *vir* helper strains, numerous different T-DNA binary vectors with specialized properties have been designed to facilitate such diverse activities as protein expression, activation tagging, protein localization, protein-protein interaction studies, and RNAi-mediated gene silencing. However, the ease of use of binary vectors may have come at a cost. The use of multicopy binary vectors generally results in integration of multiple copies of T-DNA into the plant genome. Multiple transgene copies have a propensity to silence to a greater extent than do single integrated copies. In addition, integration of vector backbone sequences from binary vectors into plant DNA, a potential regulatory problem, is common (Martineau et al., 1994; Kononov et al., 1997; Wenck et al., 1997). Integration of non-T-DNA region sequences when T-DNA is launched from large Ti-plasmids is relatively rare (Ramanathan and Veluthambi, 1995). Thus, the use of multicopy binary vectors may have exacerbated two common problems associated with plant transformation, multiple integrated transgene copy number and vector backbone integration. Launching T-DNA from low-copy-number T-DNA binary vectors or from the *Agrobacterium* chromosome may mitigate these problems (Ye et al., 2007). Such systems should greatly increase the quality of *Agrobacterium*-mediated transformation events.

ACKNOWLEDGMENTS

Work in the authors' laboratory is supported by the Biotechnology Research and Development Corporation, the Corporation for Plant Biotechnology Research, and the National Science Foundation (Plant Genome grant no. 0110023).

Received November 9, 2007; accepted November 25, 2007; published February 6, 2008.

LITERATURE CITED

Abuodeh RO, Orbach MJ, Mandel MA, Das A, Galgiani JN (2000) Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. J Infect Dis **181:** 2106–2110

An G (1987) Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol **153:** 292–305

An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) New cloning vehicles for transformation of higher plants. EMBO J **4:** 277–284

Anderson A, Moore L (1979) Host specificity in the genus *Agrobacterium*. Phytopathology **69:** 320–323

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res **18:** 203

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721

Bevan MW, Flavell RB, Chilton MD (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature **304:** 184–187

Bouchez D, Camilleri C, Caboche M (1993) A binary vector based on Basta resistance for *in planta* transformation of *Arabidopsis thaliana*. C R Acad Sci Ser III Sci Vie **316:** 1188–1193

Bulgakov VP, Kisselev KV, Yakovlev KV, Zhuravlev YN, Gontcharov AA, Odintsova NA (2006) *Agrobacterium*-mediated transformation of sea urchin embryos. Biotechnol J **1:** 454–461

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ (1995) Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. EMBO J **14:** 3206–3214

Cangelosi GA, Hung L, Puvanesarajah V, Stacey G, Ozga DA, Leigh JA, Nester EW (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. J Bacteriol **169:** 2086–2091

Cangelosi GA, Martinetti G, Leigh JA, Lee CC, Theines C, Nester EW (1989) Role of *Agrobacterium tumefaciens* chvA protein in export of beta-1,2-glucan. J Bacteriol **171:** 1609–1615

Chakrabarty R, Banerjee R, Chung SM, Farman M, Citovsky V, Hogenhout SA, Tzfira T, Goodin M (2007) pSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: probing *Nicotiana benthamiana*-virus interactions. Mol Plant Microbe Interact **20:** 740–750

Chen QJ, Zhou HM, Chen J, Wang XC (2006) A Gateway-based platform for multigene plant transformation. Plant Mol Biol **62:** 927–936

Cheng ZM, Schnurr JA, Dapaun JA (1998) Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. Plant Cell Rep **17:** 646–649

Chung SM, Frankman EL, Tzfira T (2005) A versatile vector system for multiple gene expression in plants. Trends Plant Sci **10:** 357–361

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E (2005) Biogenesis, architecture, and function of bacterial Type IV secretion systems. Annu Rev Microbiol **59:** 451–485

Citovsky V, Lee LY, Vyas S, Glick E, Chen MH, Vainstein A, Gafni Y, Gelvin SB, Tzfira T (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation *in planta*. J Mol Biol **362:** 1120–1131

Coutu C, Brandle J, Brown D, Brown K, Miki B, Simmonds J, Hegedus DD (2007) pORE: A modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Res **16:** 771–781

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiol **133:** 462–469

Day MJD, Ashurst JL, Dixon RA (1994) Plant expression cassettes for enhanced translational efficiency. Plant Mol Biol Rep **12:** 347–357

de Framond AJ, Barton KA, Chilton MD (1983) Mini-Ti: a new vector strategy for plant genetic engineering. Biotechnology (N Y) **5:** 262–269

de Groot MJA, Bundock P, Hooykaas PJJ, Beijersbergen AGM (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol **16:** 839–842

DeCleene M, DeLey J (1976) The host range of crown gall. Bot Rev **42:** 389–466

DeGreve H, Decraemer H, Seurinck J, Van Montagu M, Schell J (1981) The functional organization of the octopine *Agrobacterium tumefaciens* plasmid pTiB6S3. Plasmid **6:** 235–248

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA **77:** 7347–7351

Douglas CJ, Staneloni RJ, Rubin RA, Nester EW (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J Bacteriol **161:** 850–860

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J **45:** 616–629

Fraley RT, Rogers SG, Horsch RB, Eichholtz DA, Flick JS, Fink CL, Hoffmann NL, Sanders PR (1985) The SEV system: a new disarmed Ti plasmid vector system for plant transformation. Biotechnology (N Y) **3:** 629–635

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, et al (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA **80:** 4803–4807

Garfinkel DJ, Nester EW (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J Bacteriol **144:** 732–743

Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell **27:** 143–153

Gelvin SB (2003) *Agrobacterium* and plant transformation: the biology behind the ''gene-jockeying'' tool. Microbiol Mol Biol Rev **67:** 16–37

Gleave AP (1992) A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol **20:** 1203–1207

Goderis IJWM, De Bolle MFC, Francois IEJA, Wouters PFJ, Broekaert WF, Cammue BPA (2002) A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. Plant Mol Biol **50:** 17–27

Goodin MM, Dietzgen RG, Schichnes D, Ruzin S, Jackson AO (2002) pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. Plant J **31:** 375–383

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol **25:** 989–994

Hamilton CM (1997) A binary-BAC system for plant transformation with high-molecular weight DNA. Gene **200:** 107–116

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000a) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol **42:** 819–832

Hellens R, Mullineaux P, Klee H (2000b) A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci **5:** 446–451

Hepburn AG, White J, Pearson L, Maunders MJ, Clarke LE, Prescott AG, Blundy KS (1985) The use of pNJ5000 as an intermediate vector for the genetic manipulation of *Agrobacterium* Ti-plasmids. J Gen Microbiol **131:** 2961–2969

Herrera-Estrella L, DeBlock M, Messens E, Hernalsteens JP, Van Montagu M, Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J **2:** 987–996

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303:** 179–180

Hoekema A, Roelvink PW, Hooykaas PJJ, Schilperoort RA (1984) Delivery of T-DNA from the *Agrobacterium tumefaciens* chromosome into plant cells. EMBO J **3:** 2485–2490

Holsters M, Silva B, Van Vliet F, Genetello C, DeBlock M, Dhaese P, Depicker A, Inzé D, Engler G, Villarroel R, et al (1980) The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3:** 212–230

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res **2:** 33–50

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol **168:** 1291–1301

Hooykaas PJJ (1988) *Agrobacterium* molecular genetics. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp A4–A13

Howard EA, Zupan JR, Citovsky V, Zambryski PC (1992) The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell **68:** 109–118

Jayaswal RK, Veluthambi K, Gelvin SB, Slightom JL (1987) Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a *virD*-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J Bacteriol **169:** 5035–5045

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci **7:** 193–195

Kelly BA, Kado CI (2002) *Agrobacterium*-mediated T-DNA transfer and integration into the chromosome of *Streptomyces lividans*. Mol Plant Pathol **3:** 125–134

Klee HJ, Yanofsky MF, Nester EW (1985) Vectors for transformation of higher plants. Biotechnology (N Y) **3:** 637–642

Koekman BP, Ooms G, Klapwijk PM, Schilperoort RA (1979) Genetic map of an octopine Ti plasmid. Plasmid **2:** 347–357

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet **204:** 383–396

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector ''backbone'' sequences into the tobacco genome: Evidence for multiple complex patterns of integration. Plant J **11:** 945–957

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. Proc Natl Acad Sci USA **98:** 1871–1876

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Biotechnology (N Y) **9:** 963–967

Lee LY, Gelvin SB (2004) Osa protein constitutes a strong oncogenic suppression system that can block *vir*-dependent transfer of IncQ plasmids between *Agrobacterium* cells and the establishment of IncQ plasmids in plant cells. J Bacteriol **186:** 7254–7261

Lee LY, Humara JM, Gelvin SB (2001) Novel constructions to enable the integration of genes into the *Agrobacterium tumefaciens* C58 chromosome. Mol Plant Microbe Interact **14:** 577–579

Lee LY, Kononov ME, Bassuner B, Frame BR, Wang K, Gelvin SB (2007) Novel plant transformation vectors containing the superpromoter. Plant Physiol **145:** 1294–1300

Leemans J, Shaw C, Deblaere R, DeGreve H, Hernalsteens JP, Maes M, Van Montagu M, Schell J (1981) Site-specific mutagenesis of *Agrobacterium* Ti plasmids and transfer of genes to plant cells. J Mol Appl Genet **1:** 149–164

Li G, Zhou Z, Liu G, Zheng F, He C (2007) Characterization of T-DNA insertion patterns in the genome of rice blast fungus *Magnaporthe oryzae*. Curr Genet **51:** 233–243

Liu YG, Nagaki K, Fujita M, Kawaura K, Uozumi M, Ohigara Y (2000) Development of an efficient maintenance and screening system for large-insert genomic DNA libraries of hexaploid wheat in a transformation competent artificial chromosome (TAC) vector. Plant J **23:** 687–695

Liu YG, Shirano Y, Fukaki H, Yanai Y, Tasaka M, Tabata S, Shibata D (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc Natl Acad Sci USA **96:** 6535–6540

Luo ZQ, Farrand SK (1999) Cloning and characterization of a tetracycline resistance determinant present in *Agrobacterium tumefaciens* C58. J Bacteriol **181:** 618–626

Martineau B, Voelker TA, Sanders RA (1994) On defining T-DNA. Plant Cell **6:** 1032–1033

Matthysse AG (1995) Genes required for cellulose synthesis in *Agrobacterium tumefaciens*. J Bacteriol **177:** 1069–1075

McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol Biol **14:** 269–276

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu Rev Cell Dev Biol **22:** 101–127

Miranda A, Janssen G, Hodges L, Peralta EG, Ream W (1992) *Agrobacterium tumefaciens* transfers extremely long T-DNAs by a unidirectional mechanism. J Bacteriol **174:** 2288–2297

O'Connell KP, Handelsman J (1999) *chvA* locus may be involved in export of neutral cyclic beta-1,2 linked D-glucan from *Agrobacterium tumefaciens*. Mol Plant Microbe Interact **2:** 11–16

Ooms G, Hooykaas PJJ, Van Veen RJM, Van Beelan P, Regensburg-Tuink TJG, Schilperoort RA (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid **7:** 15–29

Palanichelvam K, Oger P, Clough SJ, Cha C, Bent AF, Farrand SK (2000) A second T-region of the soybean-supervirulent chrysopine-type Ti plasmid

pTiChry5, and construction of a fully disarmed *vir* helper plasmid. Mol Plant Microbe Interact **13:** 1081–1091

Pazour GJ, Das A (1990) Characterization of the VirG binding site of *Agrobacterium tumefaciens*. Nucleic Acids Res **18:** 6909–6913

Peng WT, Lee YW, Nester EW (1998) The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. J Bacteriol **180:** 5632–5638

Peralta EG, Hellmiss R, Ream W (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. EMBO J **5:** 1137–1142

Piers KL, Heath JD, Liang X, Stephens KM, Nester EW (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. Proc Natl Acad Sci USA **93:** 1613–1618

Ramanathan V, Veluthambi K (1995) Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of TL-DNA. Plant Mol Biol **28:** 1149–1154

Robertson JL, Holliday T, Matthysse AG (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. J Bacteriol **170:** 1408–1411

Rossi L, Hohn B, Tinland B (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA **93:** 126–130

Rothstein SJ, Lahners KN, Lotstein RJ, Carozzi NB, Jayne SM, Rice DA (1987) Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene **53:** 153–161

Saenkham P, Eiamphungporn W, Farrand SK, Vattanaviboon P, Mongkolsuk S (2007) Multiple superoxide dismutases in *Agrobacterium tumefaciens*: functional analysis, gene regulation and their influence on tumoriogenesis. J Bacteriol **189:** 8807–8817

Stachel SE, Nester EW, Zambryski PC (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc Natl Acad Sci USA **83:** 379–383

Todd R, Tague BW (2001) Phosphomannose isomerase: a versatile selectable marker for *Arabidopsis thaliana* germ-line transformation. Plant Mol Biol Rep **19:** 307–319

Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol **57:** 503–516

Tzfira T, Vaidya M, Citovsky V (2004) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature **431:** 87–92

Uberlacker B, Werr W (1996) Vectors with rare-cutter restriction enzyme sites for expression of open reading frames in transgenic plants. Mol Breed **2:** 293–295

van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res **4:** 288–290

Veluthambi K, Jayaswal RK, Gelvin SB (1987) Virulence genes *A*, *G*, and *D* mediate the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. Proc Natl Acad Sci USA **84:** 1881–1885

Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CMT, Regensburg-Tuink TJG, Hooykaas PJJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science **290:** 979–982

Vergunst AC, van Lier MCM, den Dulk-Ras A, Stuve TAG, Ouwehand A, Hooykaas PJJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proc Natl Acad Sci USA **102:** 832–837

Vilaine F, Casse-Delbart F (1987) A new vector derived from *Agrobacterium rhizogenes* plasmids: a micro-Ri plasmid and its use to construct a mini-Ri plasmid. Gene **55:** 105–114

Wang K, Herrera-Estrella L, Van Montagu M, Zambryski P (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell **38:** 455–462

Wang K, Stachel SE, Timmerman B, Van Montagu M, Zambryski PC (1987) Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium vir* gene expression. Science **235:** 587–591

Ward ER, Barnes WM (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5′ end of T-strand DNA. Science **242:** 927–930

Waters VL, Hirata KH, Pansegrau W, Lanka E, Guiney DG (1991) Sequence identity in the nick regions of IncP plasmid transfer origins and T-DNA borders of *Agrobacterium* Ti plasmids. Proc Natl Acad Sci USA **88:** 1456–1460

Wenck A, Czako M, Kanevski I, Marton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. Plant Mol Biol **34:** 913–922

Winans SC (1991) An *Agrobacterium* two-component regulatory system for the detection of chemicals released from plant wounds. Mol Microbiol **5:** 2345–2350

Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol **40:** 711–717

Xu XQ, Pan SQ (2000) An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. Mol Microbiol **35:** 407–414

Yadav NS, Van der Leyden J, Bennett DR, Barnes WM, Chilton MD (1982) Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proc Natl Acad Sci USA **79:** 6322–6326

Ye X, Gilbertson A, Peterson MW, inventors. March 29, 2007. Vectors and methods for improved plant transformation efficiency. US Patent Application No. US2007/0074314 A1

Yusibov VM, Steck TR, Gupta V, Gelvin SB (1994) Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. Proc Natl Acad Sci USA **91:** 2994–2998

Zambryski P, Joos PH, Genetello C, Leemans J, Van Montagu M, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J **2:** 2143–2150

Zupan JR, Citovsky V, Zambryski P (1996) *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. Proc Natl Acad Sci USA **93:** 2392–2397