

Binary Vectors and Super-binary Vectors

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Summary

A binary vector is a standard tool in the transformation of higher plants mediated by *Agrobacterium tumefaciens*. It is composed of the borders of T-DNA, multiple cloning sites, replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes, reporter genes, and other accessory elements that can improve the efficiency of and/or give further capability to the system. A super-binary vector carries additional virulence genes from a Ti plasmid, and exhibits very high frequency of transformation, which is valuable for recalcitrant plants such as cereals. A number of useful vectors are widely circulated. Whereas vectors with compatible selectable markers and convenient cloning sites are usually the top criteria when inserting gene fragments shorter than 15 kb, the capability of maintaining a large DNA piece is more important for consideration when introducing DNA fragments larger than 15 kb. Because no vector is perfect for every project, it is recommended that modification or construction of vectors should be made according to the objective of the experiments. Existing vectors serve as good sources of components.

Key Words: *Agrobacterium tumefaciens*; transformation; binary vector; super-binary vector.

1. Introduction

Research projects that involve transformation of higher plants are lengthy, complicated processes, which may last for years. The first parts of the projects are the steps of vector construction, performed by molecular biologists. Compared with the entire durations of the projects, these steps are relatively short and usually can be completed within weeks. However, scientists could make a series of irrevocable decisions and sometimes mistakes in these fate-determining steps, and the consequences would not emerge until years later.

Researchers often find undesired aspects of vector configuration when characterizing progeny of transgenic plants; in a worst case scenario these vector defects could ruin the entire project. In this chapter, steps from designing of vectors through preparation of strains of *Agrobacterium tumefaciens* ready for infection are addressed. It should be emphasized that good management of these processes can save a lot of precious time and resources.

Basic frameworks of the current vectors for transformation of higher plants were developed in the early and mid-1980s, soon after it had been elucidated that crown gall tumorigenesis represented the genetic transformation of plant cells (1). The first achievement was the removal of wild-type T-DNA, which causes tumors and inhibits plant regeneration, from Ti plasmids to generate “disarmed strains” such as LBA4404 (2). Earlier attempts at the introduction of engineered T-DNA into *A. tumefaciens* involved the placement of genes in *E. coli* vectors that could be integrated into a disarmed Ti plasmid (1). This was a reasonably efficient system, but a limitation was that the final product is a plasmid larger than 150 kb in *A. tumefaciens*, and confirmation of the structure was not straightforward.

Then the binary vector system was invented, exploiting the fact that the process for transfer of T-DNA is active even if the virulence genes and the T-DNA are located on separate replicons in an *A. tumefaciens* cell (2). An artificial T-DNA is constructed within a plasmid that can be replicated in both *A. tumefaciens* and *E. coli*. Plasmid construction is completed in *E. coli*, and simple transfer of the vector to *A. tumefaciens* produces a strain ready for plant transformation. Soon such binary vectors were widely distributed among plant scientists. Although the term *binary vector* literally refers to the entire system that consists of two replicons, one for the T-DNA and the other for the virulence genes, the plasmid that carries the T-DNA is frequently called a binary vector. We follow this popular and convenient terminology in this chapter.

One of the approaches toward enhancing the frequency of transformation by binary vectors is to employ additional virulence genes, such as *virB*, *virE*, and *virG*, which exhibit certain gene dosage effects (3–6). In the super-binary vector system, a DNA fragment that contains *virB*, *virC*, and *virG* from pTiBo542 is introduced into a small T-DNA-carrying plasmid (7). *A. tumefaciens* strains that carry pTiBo542 are wider in host range and higher in transformation efficiency than strains that carry other Ti plasmids, such as pTiA6 and pTiT37 (8). Super-binary vectors are highly efficient in the transformation of various plants (see **Note 1**) and played an important role when the host range of transformation mediated by *A. tumefaciens* was extended to important cereals in the mid-1990s (9,10). The final step of construction of a super-binary vector is integration of an intermediate vector with an acceptor vector in *A. tumefaciens*,

but, unlike the aforementioned integration system, the final product in the super-binary vector system is a plasmid that can be confirmed by routine restriction analysis of mini-scale DNA preparation from *A. tumefaciens*.

Commonly used binary and super-binary vectors are listed in **Table 1**. Helpful guidance for selection of the vectors has already been provided in the literature (11). Still, which is the best vector is a question with no definitive answer. Since these are vehicles for delivery of transgenes to plants, they should be (1) easy for the researcher to insert genes (loading), (2) efficient in plant transformation (unloading), (3) widely available to researchers, and (4) versatile for diverse purposes. Because such vectors can also be source materials for new vectors, if any vector components can be easily replaced or removed, the vector will be very useful. Recently constructed vectors provide a number of user-friendly features related to transgene loading and unloading, such as wide selection of cloning sites, high copy numbers in *E. coli*, high cloning capacity, improved compatibility with strains of choice, wide pool of selectable markers for plants, and high frequency of plant transformation. However, our quick survey of some 130 recently published papers, in which transformation of higher plants mediated by *A. tumefaciens* was described, revealed that derivatives of a relatively old vector, pBin19 (12), such as pBI121 (13), pIG121Hm (9), and others, were still used in about 40% of these studies. One reason could be that these vectors were widely circulated at early stages of plant transformation, and accumulated data in the literature from their use has built a lot of confidence. Another reason might be a convenient feature of pBI121, namely, that one-step replacement of the β -glucuronidase (Gus) gene with another gene can quickly create an overexpression vector for the gene.

No matter how difficult the choice is, we have to make a decision. In our laboratories, the key criteria for delivery of transgene fragments smaller than 15 kb are (1) compatibility of selectable markers with the experiments and (2) availability of convenient cloning sites. For delivering DNA fragments larger than 15 kb, the top consideration is whether the large DNA fragments can be cloned efficiently to the vectors and maintained stably in *E. coli* and *A. tumefaciens*, because large DNA pieces in certain vectors, e.g., high-copy-number vectors, may sometimes cause low efficiency of transformation of bacteria or rearrangement of the inserts (14).

The scope of transformation experiments in higher plants is complex, covering topics such as overexpression, regulated expression, downregulation or shut-down of foreign or internal genes, expression of gene fusion, assays of promoters or other regulatory elements, complementation of mutations with genomic sequences, tests of new molecular tools, tests of novel tissue culture protocols, and so on, with ever growing complexity. Therefore, in regard to the versatility of the vector, it is futile to try to design a vector that can be

Table 1

Commonly Used Binary and Super-binary Vectors

Vector	Plant selection marker	Bacterial selection marker	Source of borders	Replication origin for <i>A. tumefaciens</i>	Replication origin for <i>E. coli</i>	Mobilization	GenBank accession number, Contact	Reference,
pBin19	Kan	Kan	pTiT37	IncP	IncP	Yes	(12), U09365, michael.bevan@bbsrc.ac.uk	
pBI121	Kan	Kan	pTiT37	IncP	IncP	Yes	(13), AF485783, www.cambia.org	
pGreen series ^a	Kan, Hyg, Sul, Bar	Kan	pTiT37 (synthetic)	IncW	pUC	No	(27), www.pgreen.ac.uk	
pCambia series	Kan, Hyg	Cm, Kan	pTiC58	pVS1	ColE1	Yes	www.cambia.org	
pPZP series	Kan, Gen	Cm, Sp	pTiT37	pVS1	ColE1	Yes	(50), maliga@waksman.rutgers.edu	
pPCV001	Kan	Ap	RB: pTiC58 LB: Octopine ^b	IncP	ColE1	Yes	(54), konc@mpiz-koeln.mpg.de	
pGA482	Kan	Tc, Kan	pTiT37	IncP	ColE1	Yes	(55), genean@postech.ac.kr	
pCLD04541	Kan	Tc, Kan	Octopine	IncP	IncP	Yes	(49), AF184978, hbz.tamu.edu and www.jic.bbsrc.ac.uk/staff/ian-bancroft/vectorspage.htm	
pBIBAC series	Kan, Hyg	Kan	Octopine	pRi	F factor	Yes	(47), www.biotech.cornell.edu/BIBAC/BIBACHomePage.html	
pYLTAC series	Hyg, Bar	Kan	Octopine	pRi	Phage P1	No	(48), www.kazusa.or.jp/en/plant/TAC/index.html	
pSB11	None	Sp	pTiT37	None	ColE1	Yes	(45), AB027256, www.jti.co.jp/plantbiotech	
pSB1	None	Tc	None	IncP	ColE1	Yes	(45), AB027255, www.jti.co.jp/plantbiotech	

^aIndication of more than one marker genes for a series of vectors means availability of a vector with each one of the markers.

^bSee **Note 3**.

suitable for all purposes. It is a good idea to modify existing vectors or to build new ones from scratch for specific purposes as demands arise.

2. Materials

2.1. Components of a Binary Vector

The compositions of widely circulated binary vectors are similar, and many of the components have been used for more than 15 yr without much modification in plant transformation experiments. Therefore, the components listed in this section are considered quite reliable. It is useful to prepare series of unfinished plasmids that carry various combinations of these elements as materials for molecular construction beforehand (*see Note 2*).

2.1.1. On the T-DNA

1. T-DNA borders and their sequence contexts. These components are usually DNA fragments cloned from well-known Ti plasmids. Imperfect, direct repeats of 25 bases, the right border (RB) and left border (LB), are said to be the only essential *cis* elements for T-DNA transfer (*15*) but factors that enhance (*overdrive*) or attenuate T-DNA transfer have been identified near the RB (*16,17*) or the LB (*17*), respectively. Therefore, it may be safe to retain a few hundred bases of natural sequences adjacent to the T-DNA. Sources for the borders are also indicated in **Table 1**. Data for the border sequences are available in GenBank, accession numbers ATU237588 for pTiC58, and ATACH5 for pTi15955 (*see Note 3*).
2. Multiple cloning sites (MCS). Many of the vectors have the MCS derived from popular cloning vectors, such as pUC8/9, pUC18/19, and pBluescript, whereas others have unique sequences. The MCS from these standard vectors are convenient because gene components are usually cloned in such vectors beforehand. Some vectors have the *lacZ* unit from the standard vectors, and blue/white selection for insertion of fragments may be employed.
3. Selectable marker gene cassette for plant transformation (*see Note 4*).
 - a. Promoters. 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. Promoters used mainly for dicotyledonous plants include the 35S promoter from cauliflower mosaic virus (*18*) and promoters derived from Ti plasmids, such as nopaline synthase (Nos) (*19*), octopine synthase (Ocs), mannopine synthase (Mas), gene 1, gene 2, and gene 7 (*20*). Popular promoters for monocotyledonous plants include the 35S promoter and the promoters from the ubiquitin (Ubi) gene of maize (*21*) and the actin (Act) gene of rice (*22*). 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 (*23*; unpublished results).
 - b. Selectable markers for plants. Marker genes used in binary and super-binary vectors are listed in **Table 2**. Depending on the plants to be transformed,

Table 2

Selectable Markers (S) and Reporter Genes (R) Employed in Binary and Super-binary Vectors

Common abbreviations	Protein	S or R	Used for		Selection pressure	Ref.
			Plants	Bacteria		
NptII, Aph 3' II, Kan	Neomycin phosphotransferase II	S	Yes	Yes	Kanamycin, G418, paromomycin	(56)
Hpt, Hph, AphIV, Hyg	Aminoglycoside 3' phosphotransferase Hygromycin phosphotransferase	S	Yes	Yes	Hygromycin	(57)
NptI, Kan	Aminoglycoside phosphotransferase IV	S		Yes	Kanamycin	(14)
NptIII, Kan	Aminoglycoside phosphotransferase I	S		Yes	Kanamycin	(58)
Bar, Pat	Aminoglycoside phosphotransferase III Phosphinothricin acetyl transferase	S	Yes		Phosphinothricin (BASTA), bialaphos	(59)
PMI	Phosphomannose isomerase	S	Yes		Mannose as sole carbon source	(60)
Ble	Bleomycin binding protein	S	Yes		Bleomycin, phleomycin	(61)
Sul	Mutant dihydropteroate synthase	S	Yes		Sulfonamide	(62)
BSD	Blasticidin deaminase	S	Yes		Blasticidin S	(63)
Als	Mutant acetolactate synthase	S	Yes		Sulfonyleurea, imidazolinone, bispribac-sodium	(64)
AHAS	Mutant acetohydroxy acid synthase	S	Yes		Chlorsulfuron	(65)
DHFR	Dihydroforate reductase	S	Yes		Methotrexate	(66)
Gen	Gentamycin acetyltransferase	S	Yes	Yes	Gentamycin	(67)
EPSP	5-Enolpyruvylshikimate-3-phosphate synthase	S	Yes		Glyphosate (Round-up)	(68)
Ipt	Isopentenyl transferase	S	Yes		Cytokinin free	(69)
AadA, SPT, Spec	Aminoglycoside-3"-adenyltransferase Aminoglycoside nucleotidyl transferase	S	Yes	Yes	Spectinomycin, streptomycin	(70)
CAT, Cm	Chloramphenicol acetyltransferase	S, R	Yes	Yes	Chloramphenicol	(71)
Bla, Amp, Carb	β -Lactamase	S		Yes	Ampicillin, carbenicillin	(14)
Tet, TetA, TC	Tetracycline efflux protein	S		Yes	Tetracycline	(14)
Cah	Cyanamide hydratase	S	Yes		Cyanamide	(72)

Tdc	Tryptophan decarboxylase	S	Yes	4-Methyl tryptophan	(73)
XylA	Xylose isomerase	S	Yes	D-Xylose as sole carbon source	(74)
hemL, GSA-AT	Mutant glutamate-1-semialdehyde aminotransferase	S	Yes	Gabaculine	(75)
TtdA, DPAM	2,4-Dichlorophenoxyacetate monooxygenase	S	Yes	2,4-D	(76)
Bxn	3,5-Dibromo-4-hydroxybenzoic acid nitrilase	S	Yes	Bromoxynil	(77)
Pflp	Ferredoxin-like-protein	S	Yes	<i>Erwinia carotovora</i>	(78)
PPO	Mutant protoporphyrinogen oxidase	S	Yes	Butafenacil (herbicide)	(79)
DOGR	2-Deoxyglucose-6-phosphate phosphatase	S	Yes	2-Deoxyglucose	(80)
Gus, UidA	β -Glucuronidase	R	Yes		(13)
Luc	Luciferase	R	Yes		(33)
GFP	Green fluorescent protein ^a	R	Yes		(32)
LacZ	β -Galactosidase	R	Yes	Yes	(81)
Nos	Nopaline synthase	R	Yes		(82)
R-nj	Anthocyanin	R	Yes		(83)
OxO	Oxalate oxidase	R	Yes		(84)

^aA number of useful, distinctive derivatives of GFP and fluorescent proteins with different characteristics are available and reviewed in the literature (85).

the choice of selectable markers greatly affects the efficiency of transformation, and restrictive/permissive concentrations of selective agents vary considerably among plant species and even among cultivars (see **Note 5**). Kanamycin resistance is good for many dicotyledons including tobacco, tomato, potato, and *Arabidopsis* (see **Note 6**). Hygromycin resistance (*hpt*) is very good for rice transformation (**9**), and the phosphinothricin resistance gene (*bar*) is efficient for maize and other cereals (**10,24**). We do not think the effects of selectable markers have been explored sufficiently, because many of them were tested in very limited species of plants. Therefore, we made a comprehensive list of marker genes in **Table 2**, hoping that some of the markers may be investigated further to improve transformation of certain plant species.

- c. 3' Signal (see **Note 7**): DNA fragments of a few hundred bases derived from the 3' ends of the CaMV 35S transcript and *Agrobacterium* Nos and other T-DNA genes are carried by many of the binary and super-binary vectors.

2.1.2. On the Vector Backbone

1. Bacterial selectable marker gene. 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* (**Table 2**). 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* (see **Note 8**). If such a dual function gene is present in the T-DNA, bacterial selectable markers may be omitted from the vector backbone to simplify the vector construction.
2. Plasmid replication functions. Binary vectors need to be replicated both in *E. coli* and *A. tumefaciens*. Use of plasmid replication functions with a wide host range, such as the ones of plasmid incompatibility group P (IncP) or W (IncW) is a good option. IncP binary vectors carry the origin of vegetative replication (OriV) and the transacting replication functions (Trf) of IncP plasmids (**25**). The replication locus of IncW plasmids, such as pSa, consists of the origin of replication and RepA gene (**26**). pGreen vectors have only the origin of replication, and the RepA function is provided by another plasmid, pSoup, in *A. tumefaciens* (**27**). Alternatively, replication functions for *A. tumefaciens*, such as the ones for an Ri plasmid (**28**) or pVS1 (**29**), and for *E. coli*, such as the ones for the F factor, phage P1, ColE1, or P15A (**14**), may be combined. Types of replication functions determine copy numbers and stability of the plasmids in bacterial cells. The use of high-copy plasmids for cloning of fragments larger than 15 kb can sometimes result in complications like rearrangement of DNA. If this is a problem, a good choice is an IncP plasmid, such as pBI121, which is a low-copy-number (about five copies per cell) plasmid in both *E. coli* and *A. tumefaciens* (see **Note 9**).
3. Plasmid mobilization functions (see **Note 10**). The origin of transfer (OriT) of IncP plasmids (**25**) or the bom function of ColE1 plasmid (**14**) is carried by most

of the binary vectors. Plasmids with one of the sequences may be mobilized from *E. coli* to *A. tumefaciens* aided by a conjugal helper plasmid, pRK2013 or pRK2073 (30). pRK2073 is a derivative of pRK2013 and has an insertion of Tn7 in the kanamycin resistance gene of pRK2013. Either one works fine for most applications, but pRK2073 is recommended if the target plasmid carries kanamycin resistance, and pRK2013 is recommended if the target has spectinomycin resistance.

2.2. Components of a Super-binary Vector

2.2.1. Intermediate Vector

1. T-DNA. The same composition described for a binary vector applies (see **Subheading 2.1.1.**).
2. Plasmid replication. An intermediate vector has an origin of replication of ColE1 like pBR322 and is replicated in *E. coli* but not in *A. tumefaciens*.
3. Plasmid mobilization. An intermediate vector has the bom function of ColE1 near the replication origin and can be mobilized by pRK2013 or pRK2073.
4. Bacterial selection. An intermediate vector has a spectinomycin resistance gene derived from Tn7 (31).
5. Homology with an acceptor vector. An intermediate vector and an acceptor vector share the 2.7-kb fragment that contains the ori and bom of ColE1 and the cos site from phage lambda (14) (the Ori-Cos fragment).

2.2.2. Acceptor Vector

1. Plasmid replication. An acceptor vector is an IncP plasmid and also has the ori of ColE1.
2. Plasmid mobilization. An acceptor vector has both the bom function of ColE1 and OriT of IncP plasmids and can be mobilized aided by pRK2013 or pRK2073.
3. Bacterial selection. An acceptor vector has tetracycline resistance derived from pRK2.
4. Virulence genes. An acceptor vector has a 14.8-kb *KpnI* fragment (the super-vir fragment) from pTiBo542. This fragment contains *virB*, *virC*, and *virG* operons (see **Note 1**).
5. Homology with intermediate vectors (see **Subheading 2.2.1.**).
6. T-DNA with a plant-selectable marker gene. An acceptor vector optionally has a T-DNA to create a vector for cotransformation.

2.3. Reporter Gene Cassette

1. Promoters. 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.

2. Reporter genes. β -Glucuronidase (Gus) (13), green fluorescent protein (GFP) (32) and luciferase (Luc) (33) are the most popular reporter genes (Table 2). 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. Because Gus and GFP are highly stable proteins in plant cells (34), 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. If this is the case, Luc, whose half-life in plant cells is much shorter than those of Gus and GFP (34), may be a good choice. A reporter gene that has an intron in the coding sequence, such as the *intron-Gus* gene (35), is very useful because this gene is not expressed in *A. tumefaciens*. In addition, especially in monocotyledons, introns can enhance expression for some genes (36). Introns placed close to the N-terminal in the coding sequence and in the 5' untranslated region of a gene may be equally effective (37).
3. 3' Signal. The 3' signal for a reporter gene may be chosen from the components listed for the selectable marker genes (see Subheading 2.1.1.).

2.4. Accessory Components for Binary and Super-binary Vectors

1. Restriction sites for endonucleases with long recognition sequences. Because genes have various restriction sites, it may not always be easy to find unique sites for introducing DNA sequences to a desired location on a vector. More than 10 restriction enzymes that recognize 8 bases are available now, and there are several homing endonucleases, which have recognition sites longer than 10 bases. It is useful to design vectors with a number of sites for these enzymes. Such vectors are especially useful for stacking of multiple expression units in one vector. Well-designed sets of plasmids that consists of a binary vector with these sites and high-copy cloning vectors with expression cassettes and subsets of the sites are called as *modular vectors* (38).
2. The sites for the GATEWAY® system. Molecular cloning based on restriction enzymes and DNA ligases is not always straightforward. The GATEWAY system (Invitrogen) is a cloning technology based on the site-specific recombination system of phage lambda. A step of molecular cloning may be performed in a single tube within a few hours, and *E. coli* that carries a desired plasmid is recovered at a very high frequency on the following day. In essence, a DNA fragment flanked by a pair of short, specific sequences may easily be replaced with another DNA fragment by the GATEWAY system. By placing the GATEWAY recombination sites at appropriate locations in the vectors, workload for subsequent cloning steps may be greatly reduced. Convenient binary vectors based on the GATEWAY technology have been reported by various authors (39,40).
3. Virulence genes. Small DNA fragments that contain *virE* or *virG* can improve the efficiency of transformation by a binary vector to some extent (4,5). A mutant *virG* gene, *virGN54D*, that is expressed constitutively in *Agrobacterium* cells gave much higher efficiency of transformation than wild types (41,42).

4. Device to suppress transfer of non-T-DNA segments.
 - a. Multiple left border repeats. Transfer of so-called “backbone sequences” from binary vectors to higher plants is not uncommon and has raised considerable concerns over genetically modified plants. A simple method is to place additional LB sequences close to the original LB; transfer of the backbone sequences is then suppressed in a nearly perfect fashion (43).
 - b. Killer gene. Another method is to place a gene, whose gene product is lethal to cells, outside the T-DNA to eliminate transformed cells that acquired the backbone (44).
5. Cosmid. The cloning capacity for a cosmid is up to about 50 kb, which includes the vector DNA, being based on the packaging system of phage lambda (14). A simple addition of one or two copies of cos sites can convert a binary vector to a cosmid.
6. P1 Cloning system. A P1 vector is more complex than a cosmid, and several components need to be integrated. However, P1 vector is very useful in genomic studies because the cloning capacity for a P1 vector is as large as 100 kb, being based on the packaging system of phage P1 (14).
7. Device for removal of selectable marker genes.
 - a. Cotransformation. Considerable concern has been raised over selectable marker genes in commercial transgenic plants. Cotransformation with two separate T-DNAs is a simple approach for removal of the marker gene. One T-DNA carries a selectable marker gene, and the other does genes of interest. There is a good chance that these T-DNAs, segregate independently, and marker-free progeny plants are identified. Two T-DNAs may easily be constructed on a super-binary vector (45). The only modification is that a T-DNA with a selectable marker gene is cloned into a precursor of an acceptor vector before the virulence fragment is inserted and an intermediate vector is prepared without a selectable marker gene.
 - b. Site-specific recombination systems. Many authors have reported vectors exploiting site-specific recombination systems derived from phages or fungi, such as the Cre-lox, Flp-FRT, and R/RS (46). In such a vector, a marker gene is flanked by the short target DNA sequences for a specific recombinase. After the integration of the T-DNA to plant cells, the recombinase is provided to the cells by various sophisticated means so that the marker gene is excised out.
8. Accommodation for very large DNA segments. For map-based cloning of plant genes, complementation tests of large genomic fragments provide key information. Single-copy vectors that carry Ri ori for *A. tumefaciens* and F ori or P1 ori for *E. coli* were specifically designed for transfer of very large DNA fragments to higher plants and designated as BIBAC (47) and TAC (48). On the other hand, because a simple IncP binary vector was able to maintain DNA fragments stably over 300 kb (49), conventional binary vectors that do not carry the ori of ColE1 or pUC may also be good for this purpose. We think the current situation is that cloning and transfer of DNA fragments larger than 50 kb is possible, but the efficiency is still low with any of these vectors.

3. Methods

3.1. Construction of a Typical Binary Vector

A standard flowchart showing the construction of binary vectors, from various components to the creation of an *empty vector*, a vector with a plant-selectable marker (*selection vector*), and finally a vector with both a reporter gene and a selectable marker (*reporter vector*) is illustrated in **Fig. 1**. The reporter gene in this flowchart may be a model for any genes of interest. In transformation experiments, a reporter vector may serve as a control vector, which gives reference points for virtually all important measurements in transformation processes, such as frequency of transformation, growth of transformed cells, efficiency of plant regeneration, growth of transgenic plants, phenotypes of plants, level of foreign gene expression, effects of genes of interest, and so on (see **Note 11**). Our recommendation is to start the consideration of experimental designs from the configuration of such a control vector. Quite often, a reporter vector may be a good starting material for various gene constructs, as experimental vectors may be obtained simply by replacing one or more components in the reporter vector with appropriate DNA fragments. Useful tips related to vector construction are given in **Notes 12–18**.

1. Obtain plasmids and other DNA fragments necessary for constructions of vectors from appropriate sources.
2. Combine the bacteria-selectable marker and the plasmid replication functions for *E. coli*.
3. Insert the plasmid replication functions for *A. tumefaciens*, if necessary.
4. Insert the plasmid mobilization functions, if necessary.
5. Insert the RB, the LB, and the MCS to give the empty vector.
6. Construct the expression unit of the selectable marker gene separately.
7. Insert the unit into the empty vector to give the selection vector.
8. Construct the expression unit of the reporter gene separately.
9. Insert the unit into the selection vector to give the reporter vector.

3.2. Examples of Binary Vectors

Some examples of binary vectors are shown in **Fig. 2**. Various derivatives differing in the selectable marker, reporter, MCS, and other factors are available in the pCambia series (www.cambia.org), pGreen series (27), and pPZP series (50). One of the derivatives is shown for each of the groups; empty vector pGreen0000, selection vector pPZP111, and reporter vector pCambia1302. A similar variation is found in the derivatives of pBin19, which is considered to be a selection vector, and reporter vector pBI121, shown in **Fig. 2**. An empty version of pBin19 may be obtained by digesting pBin19 with *Cla*I and partially with *Sac*II, followed by recircularization. These vector groups have been

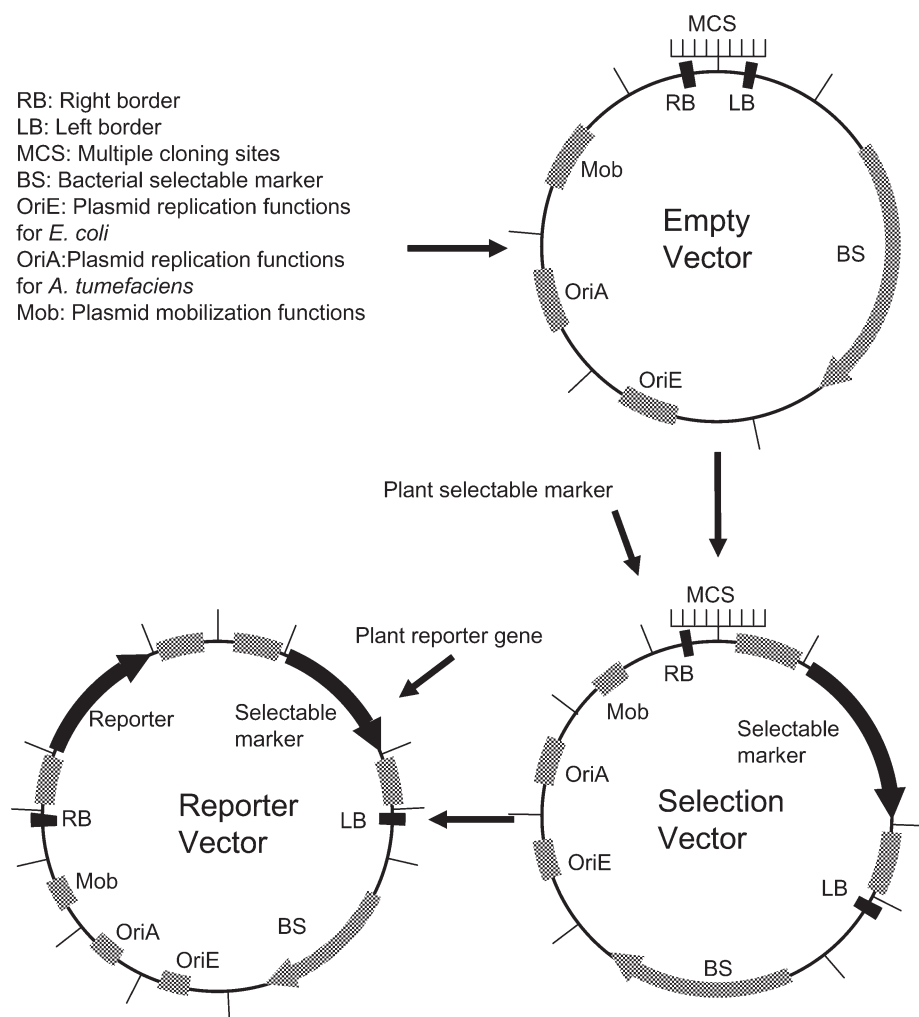


Fig. 1. A simplified flowchart showing the construction of binary vectors.

successfully employed in many studies, which, at a glance, account for two-thirds of the recent publications in the area of plant transformation.

3.3. Construction of a Typical Super-binary Vector and Examples

The system of a super-binary vector consists of two plasmids, an intermediate vector and an acceptor vector, and the final construct is a cointegrate plasmid created by homologous recombination in *A. tumefaciens* (45). Protocols for both a single T-DNA vector, in which a selectable marker and a gene of

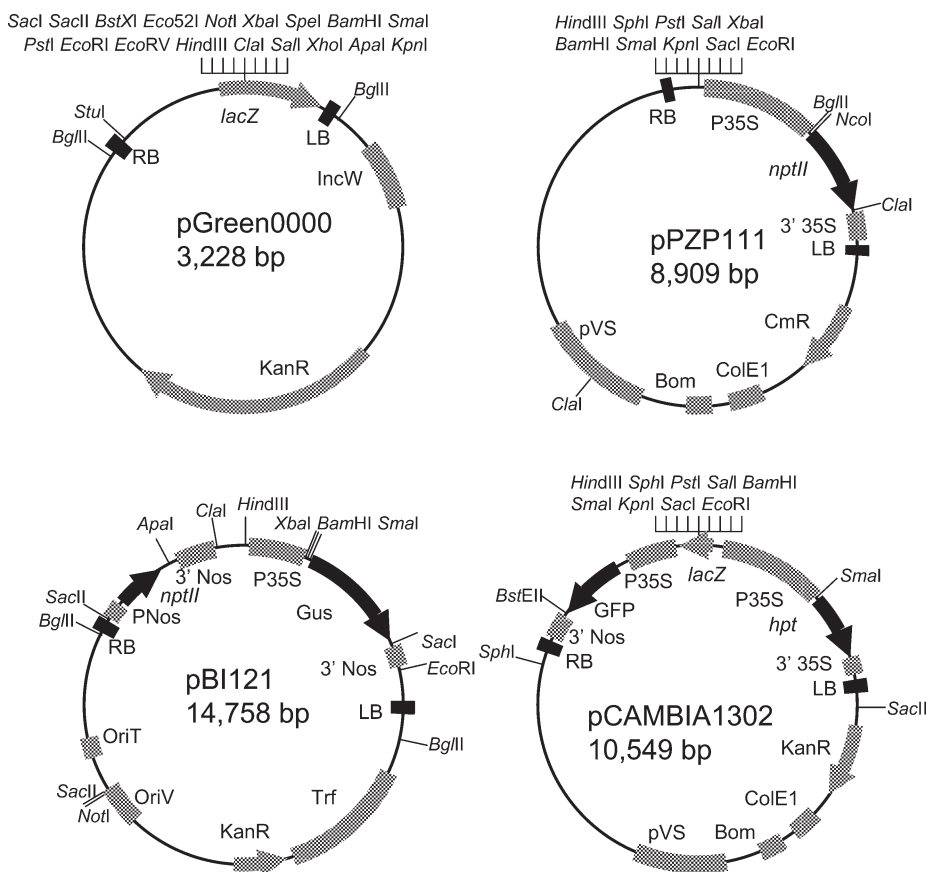


Fig. 2. Examples of binary vectors. The maps are based on sequences in GenBank, accessions numbers CVE7829 for pGreen0000, CVU10487 for pPZP111, AF485783 for pBI121, and AF234298 for pCambia1302. Abbreviations: RB, right border; LB, left border; P35S, promoter for 35S transcript; 3' 35S, 3' signal for 35S transcript; PNos, promoter for nopaline synthase; 3' Nos, 3' signal for nopaline synthase; *nptII*, neomycin phosphotransferase II; *Gus*, β -glucuronidase; *hpt*, hygromycin phosphotransferase; *lacZ*, α -subunit of β -galactosidase; *IncW*, origin of replication of *IncW* plasmid; *pVS*, origin of replication of *pVS1*; *OriV*, origin of vegetative replication of *IncP* plasmid; *Trf*, transacting replication function of *IncP* plasmid; *OriT*, origin of transfer of *IncP* plasmids; *ColE1*, origin of replication of *ColE1*; *Bom*, bom site for plasmid transfer of *ColE1*; *CmR*, chloramphenicol resistance gene; *KanR*, kanamycin resistance gene.

interest are linked in a T-DNA, and a double T-DNA vector for cotransformation are described here.

3.3.1. Single T-DNA Vector

1. Construct an empty intermediate vector by combining the Ori-Cos fragment, the spectinomycin resistance gene from Tn7, the cos site of phage lambda, the RB and the LB from pTiT37, and the MCS. pSB11 (**Fig. 3**) is an example.
2. Clone a gene of interest and a plant-selectable marker gene into the MCS of the empty vector.
3. As a preliminary step in the construction of an acceptor vector, combine the Ori-Cos fragment, the tetracycline resistance locus, OriV, Trf, OriT, and an MCS that consists of *Xba*I, *Sac*I, *Xho*I, *Kpn*I, and *Hind*III recognition sites to give a precursor plasmid. pNB1 is an example (**Fig. 3**).
4. Clone the Super-vir fragment into the *Kpn*I site of the precursor plasmid to give an acceptor vector (*see Note 19*). pSB1 (**Fig. 3**) is an example.
5. Introduce the derivative of the intermediate vector into an *A. tumefaciens* strain that carries the acceptor vector by triparental mating, and select a strain for spectinomycin and tetracycline resistance and growth on a minimal medium to create the cointegrate (*see Note 20*).

3.3.2. Double T-DNA Vector

1. Clone separately a gene of interest and a plant-selectable marker gene into an empty intermediate vector.
2. Cut out the T-DNA of the selectable marker from the intermediate vector as a *Sal*I fragment and clone into the *Xho*I site of the precursor plasmid described in **Subheading 3.3.1.** (*see Note 21*).
3. Clone the Super-vir fragment into the derivative of the precursor plasmid to create an acceptor vector with the plant-selectable marker gene. pSB4U (**Fig. 2**) (**43**), which has the hygromycin resistance gene connected to the Ubi promoter and the Nos 3' signal, is an example.
4. Introduce the intermediate vector with the gene of interest into an *A. tumefaciens* strain that carries the acceptor vector with the plant-selectable marker gene by triparental mating and select a strain for spectinomycin and tetracycline resistance and growth on a minimal medium to create the cointegrate.

3.4. Preparation of *A. tumefaciens* with Binary and Super-binary Vectors Ready for Infection

After completion of molecular construction in *E. coli*, vectors are transferred to *A. tumefaciens* by the procedures described in Chapter 3 of this volume. For the construction of super-binary vectors, triparental mating is highly recommended (*see Note 20*).

Rearrangement of vectors may sometimes take place during the process of introduction of plasmids into *A. tumefaciens*. It is very important to confirm

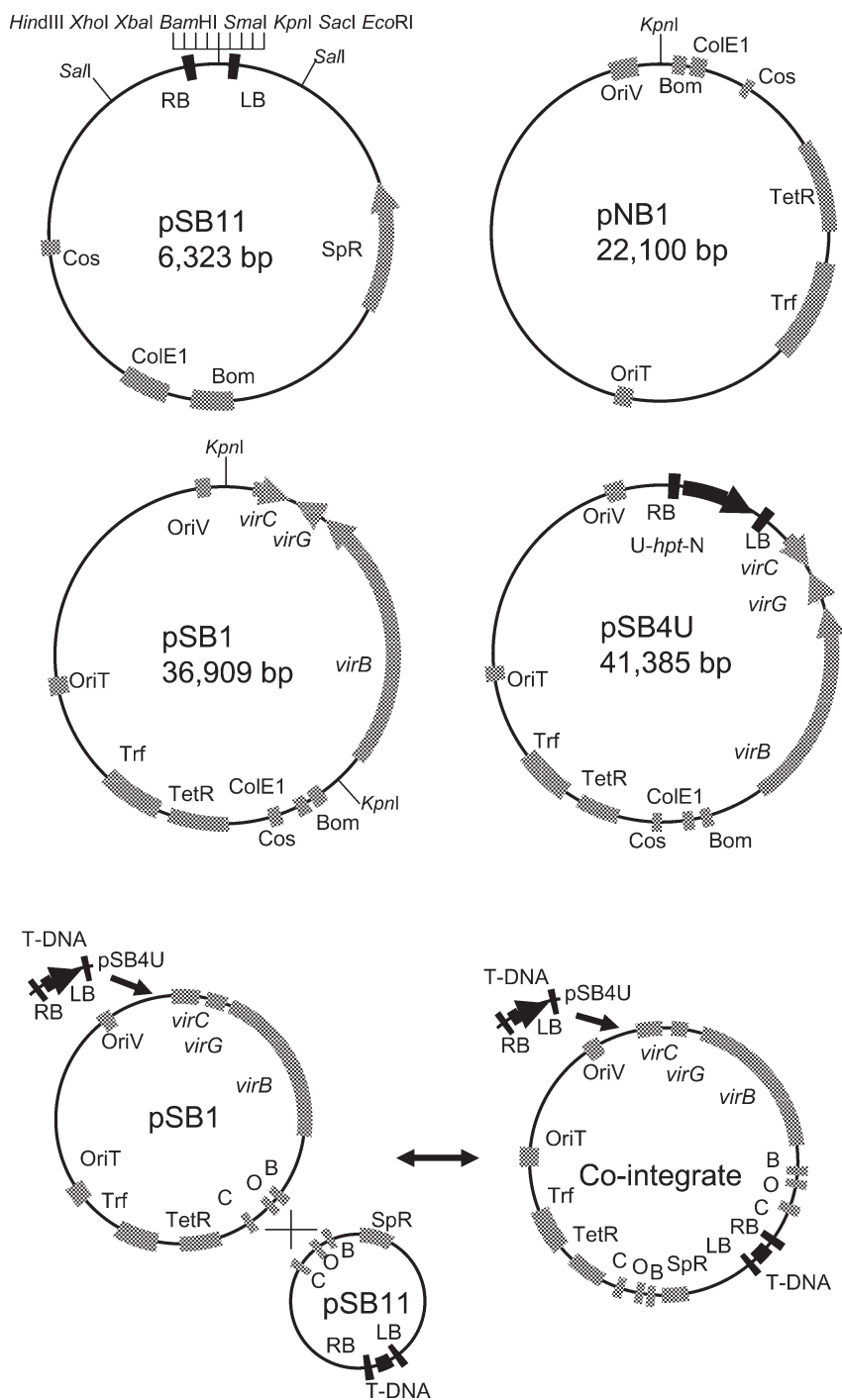


Fig. 3.

the structure of vectors in colonies of *A. tumefaciens* and select the colonies that carry the right vectors. Once the right colonies are identified, the vectors are reasonably stable in *A. tumefaciens*.

Ideally, plasmids are purified from *A. tumefaciens* and extensively characterized by restriction analysis. However, preparation of plasmids from *A. tumefaciens* is much less efficient than that from *E. coli*, and a certain amount of experience with this protocol is needed to obtain good preparations of the plasmids. Alternatively, amplification of a number of key fragments of the vectors by polymerase chain reaction (PCR) from the colonies of *A. tumefaciens* or Southern analysis of total DNA preparation from *A. tumefaciens* cells is performed.

If something is wrong with the transformation vector, years of time, effort, and precious resources can be wasted. A laboratory should establish a series of quality control (QC) protocols for vectors and bacterial strains (*see* **Note 22**). Ideally, QC protocols are written down, and a member of the laboratory is designated as the QC manager, who makes sure everyone follows the rules of the laboratory.

4. Notes

1. The capability of a super-binary vector is most evident when it is combined with strain LBA4404, whereas the performance of a super-binary vector is not very good when it is carried by strains derived from A281, such as EHA101, EHA105, or AGL1 (6,9). The *virC1* gene in the super-binary vector is probably inactive owing to a frame-shift mutation that took place during the construction of vectors (see GenBank accession number AB027255), but there is no evidence that this affects the efficiency of transformation.
2. Useful examples of the plasmids are listed below, but do not try to make a complete set at one time. It is good enough to create plasmids as required and let the library grow over time.
 - a. Minus-one vector: this vector lacks one of the components of the expression units of the vector.
 - b. Empty vector: the T-DNA of this vector has only MCS.

Fig. 3. (*previous page*) Examples of super-binary vectors and illustration of integration of an intermediate vector to an acceptor vector. The maps of pSB11, pSB1, and pSB4U are based on GenBank accession numbers AB027256, AB027255, and AB201314, respectively. Abbreviations: RB, right border; LB, left border; U-*hpt*-N, Ubiquitin promoter-hygromycin phosphotransferase-3' signal for nopaline synthase; OriV, origin of vegetative replication of IncP plasmid; ColE1 or O, origin of replication of ColE1; Trf, transacting replication function of IncP plasmid; OriT, origin of transfer of IncP plasmid; Bom or B, bom site for plasmid transfer of ColE1; Cos or C, Cos site of phage lambda; TetR, tetracycline resistance gene; SpR, spectinomycin resistance gene.

- c. High-copy MCS vector: a high-copy-number plasmid that has only MCS and no T-DNA borders. This may be convenient for creation of construction intermediates, especially in case the vector to be constructed is a low-copy-number plasmid
 - d. Marker cassette vector: the high-copy MCS vector that carries the expression unit of the plant-selectable marker gene.
 - e. Reporter cassette vector: the high-copy MCS vector that carries the expression unit of the reporter gene.
3. The T-DNA border regions of nopaline-type plasmids, pTiC58 and pTiT37, are almost identical, and the T-DNA sequences of the well-studied octopine-type Ti plasmids, pTi15955, pTiAch5, pTiA6, and pTiB6S3, are very similar to each other if not identical (51,52).
 4. In vectors constructed earlier, the selectable markers were located close to the RB. Because the transfer intermediate of the T-DNA is made in the direction from the RB to the LB, it is considered that deletion of a gene of interest may be prevented by placing a selectable marker close to the LB, and later most plasmids were constructed in this way. In contrast, integration of T-DNA into a plant chromosome is said to take place in the direction from the LB to the RB (53). We have observed deletions at both the right and left ends of the T-DNA in plants. Therefore, it is our opinion that the location of the selectable marker does not matter much.
 5. The use of weak promoters may not always be a bad idea. Strong promoters could waste resources for transcription and translation machinery in plant cells after transformation. In addition, because the levels of expression of marker genes and genes of interest are often linked, selection of transformants with weak selectable markers may cause strong expressers of the genes of interest to be obtained.
 6. Expression of the *nptII* gene can inactivate a group of aminoglycoside antibiotics (14). Choice of antibiotic is an important factor in plant transformation. For example, because kanamycin does not restrict growth of rice and maize cells, it is not used for transformation of these plants. Many transformed rice cells resistant to G418 were albinos. However, rice and maize can be transformed reasonably well with the *nptII* gene based on resistance to paromomycin (unpublished results).
 7. The DNA segments connected to the 3' ends of genes are often called terminators, but the terminology is sometimes confusing, because signals for the termination of transcription and for the addition of polyA sequences are different. Exact functions of many 3' sequences are not well characterized and it is not usually confirmed whether termination signals are really contained in terminators. Therefore, we use the term 3' signal in this chapter.
 8. Partly because the TATA boxes of eukaryotic promoters resemble prokaryotic promoters to some extent, many plant promoters are active in both *E. coli* and *A. tumefaciens* (35). The fact that both the widely used strain EHA101 and the vector pBin19 have kanamycin resistance had caused some inconvenience in earlier days. One solution was insertion of the 35S-*hpt* gene to pBin19, which gave

another resistance to the vector and made introduction of the vector to the strain easier.

9. Subcloning of a gene into IncP plasmids can sometimes be a less efficient practice compared with that of using the pUC derivatives. It is advisable to follow faithfully the cloning procedures and tips described in standard textbooks such as *Molecular Cloning* (14). Oversimplification or too many shortcuts during the subcloning process may lead to complications and delay the completion of construction. Sequence and other genetic information of IncP plasmids has been previously described (25). In general, IncP vectors that are larger than 20 kb can be more stably maintained in both *E. coli* and *A. tumefaciens* than smaller IncP vectors. We have observed that, after 3 d of culture without selective antibiotics, most of the *A. tumefaciens* cells retained large IncP plasmids, whereas more than half of the cells lost the small IncP vectors. How this phenomenon affects the transformation experiments is not known. This can be important for *Arabidopsis in planta* transformation, in which *A. tumefaciens* cells probably sit in plant tissues for some time before the gene transfer process takes place.
10. Although plasmid mobilization functions are not needed for transformation of *A. tumefaciens* by electroporation or freeze-thaw methods, it is a good idea to have broader options. When plasmid cointegration in *A. tumefaciens* is intended, triparental mating is much more efficient than electroporation, and these functions are necessary.
11. When plants are transformed with various gene constructs, it is a good idea to always transform plants in parallel with such a vector, which is extremely useful in monitoring many aspects of transformation processes. Many factors are involved in successful transformation, and it is not a simple task to maintain capability of plant transformation stably over time. If something goes wrong in experiments, what is happening in the control plots can answer many questions. It is a good idea to include the same control vector in all the transformation experiments conducted in a laboratory. We have been transforming rice for more than 15 years now with *A. tumefaciens*. We can still compare data between current and very early experiments if pIG121Hm (9) is included. Such a control vector could also play the role of a “spearhead”. New enabling technologies are continuously developed, and new methods for plant transformation are tested one after another. Each time, new factors are incorporated into mainstream protocols in a laboratory after they are tested extensively with the control vector.
12. Do not assume that external information related to biological materials is 100% correct. This is one of the hot spots for complications. You may receive incorrect or incomplete maps, sequence information, protocols, and even plasmids. The earlier you confirm materials and information after receipt, the more easily problems are identified and solved.
13. Characterize biological materials including plasmids and DNA fragments, and evaluate data collected in-house and external information as extensively as practical. Ideally, everything from external sources is fully sequenced in-house. At least, partial sequencing of the most critical segments and restriction analysis

with every six-base cutter enzyme available should be performed. The finished vectors should also be characterized as described here.

14. Simulate vector construction *in silico* and prepare sequence files and maps of the vectors to be constructed before starting wet laboratory practices.
15. All fragments amplified by PCR must be fully sequenced.
16. It should be noted that similar genes, which consist of the same promoters, the same coding sequences, and the same 3' signals, could still be expressed quite differently even in the same plant species when they are placed in different vectors, probably being affected by small differences in the configurations of the vectors. Because the nature of these effects is not well understood, it is a good idea to consider more than one molecular design and to use trial and error.
17. A prudent approach for experimental constructs is to make as few alterations as possible from a reporter vector, from which marker genes have been expressed very well.
18. In the design of vectors, avoidance of repeats of sequences is highly recommended.
19. The packaging extracts from phage lambda can greatly facilitate this cloning because the precursor is a cosmid and the size of the precursor plus the Super-vir fragment is good for the packaging reaction.
20. Triparental mating (*see* Chapter 3) is a simple technique in principle but is sometimes a hot spot of complications. The selection of *A. tumefaciens* from *E. coli* is often based on capability of growth on a minimal medium. A certain background growth of nontarget bacteria is inevitable on primary selection plates, but colonies that can grow as fast on the selective medium as on a nonselective medium are clearly distinguishable. Second selection plates are usually clean, but it is a good idea to perform one more selection culture. After the third selection, plasmids are prepared from the selected colonies as described in Chapter 3 in this volume, and restriction analysis is performed.
21. Although there are unique restriction sites in pSB1 and other acceptor vectors, direct cloning of additional DNA fragments into these vectors is not efficient. Therefore, if modification of an acceptor vector is necessary, it is a good idea to go back one step to a precursor plasmid like pNB1, which lacks the Super-vir fragment. After modification of the precursor plasmid, the 14.8 *Kpn*I Super-vir fragment is cloned back. Again, the packaging extracts from phage lambda can facilitate this cloning. It should also be noted that pUC plasmids carrying the Super-vir fragment are sometimes unstable, and pSB1 is a good source of this fragment.
22. The following points should be addressed in standard QC protocols:
 - a. Source materials: it is a good idea to establish a central stock of a laboratory of commonly used plasmid DNA, bacteria, and other biological materials used for construction of vectors.
 - b. Constructed vectors and strains: DNA of finished vectors and all construction intermediates should be stored for a defined length of time. Purified plasmid DNA may be stored at -20°C for a very long time. Key plasmids are stored as DNA and *E. coli* with the plasmids.

- c. Finished vector: a standard process for quality checking of vectors ready for plant transformation should be established. Thorough restriction analysis with more than 10 enzymes and sequencing of junctions of fragments manipulated during the construction are minimum requirements. Important vectors should be fully sequenced.
- d. *A. tumefaciens* strain ready for transformation: the routine practice for confirmation of structures of vectors in *A. tumefaciens* and the method of storage of the strains should be described.
- e. Handling of *A. tumefaciens*: proper handling of bacterial strains recommended by bacteriologists may not always be exercised by current molecular biologists. For *E. coli*, which outgrows virtually any organisms in laboratories, this is usually not a problem, but for *A. tumefaciens*, practices like always using fresh, well-isolated colonies, whose shapes and colors are carefully examined, are important.
- f. PCR primers and probes: quite a few pairs of primers and probe fragments are used during the construction. These are also useful for confirmation of vector structure and analysis of transgenic plants. It is a good idea to organize a library of these materials in a laboratory.
- g. Bioinformatics: sequences, maps, and other data from various assays of the aforementioned materials constitute an enormous amount of electronic data, which should be well organized, regularly updated, and available to everybody in the laboratory.

Acknowledgments

We are grateful to Dr. Michael Bevan, Dr. Pal Maliga, Dr. Richard Jefferson, Dr. Csaba Koncz, Dr. Gynheung An, and Dr. Hongbin Zhang for agreeing to be contacts for the vectors listed in Table 1. We thank Dr. Kan Wang for helpful discussions and encouragement and Ms. Kumiko Donovan and Ms. Ayaka Yamashita for technical assistance.

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Agrobacterium Protocols

Volume I

Wang, K. (Ed.)

2006, XXII, 484 p. 89 illus., 6 illus. in color., Hardcover

ISBN: 978-1-58829-536-1

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