Minimal T-DNA vectors suitable for agricultural deployment of transgenic plants

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Transgenic plants have become an important research tool for both plant science research and the development of new elite cultivars for use in agriculture. *Agrobacterium*-mediated gene transfer is well-recognized as the preferred approach for genetic engineering of plants. For this purpose a wide range of binary vector systems with versatile transfer DNA (T-DNA) regions have been constructed to facilitate the development of transgenic plants (1). A major limitation of most vectors is the limited number and choice of unique restriction sites in the multiple cloning site for insertion of target genes. Exceptions are pBINPLUS (2) and pGreen (3), with 12 and 16 unique sites, respectively. However, many vectors contain extraneous DNA elements on the T-DNA region destined to be transferred to plants. Such DNA often includes origins of plasmid replication, *lacZ'* cloning regions, reporter genes, and/or bacterial marker genes (2–8). Even vectors proposed as concise or clean systems carry a *lacZ'* region (7,8). Furthermore, DNA sequencing of T-DNA regions of other vectors often reveals partial DNA fragments of such regions resulting from previous cloning events that remain as artifacts not recorded on plasmid maps. Extraneous DNA regions are present as a matter of convenience rather than of necessity for the desired transgenic plant. For the general release of transgenic plants into agricultural production, such extraneous DNA regions either necessitate additional risk assessment or may be unacceptable to regulatory authorities (9). Some transgenic crops in current commercial use around the globe can be traced back to transformation events using poorly designed vectors with T-DNA regions containing bacterial

selectable marker genes and origins of plasmid replication (e.g., cotton event MON1445; see database held at www.agbios.com). For the use of transgenic plants in agriculture, it is important that minimal T-DNA vectors with no extraneous DNA segments on the T-DNA are designed and built.

The first minimal T-DNA vector, pSR8-30 (10), had two deficiencies. First, the multiple cloning region for insertion of agriculturally useful genes was located between the left T-DNA border and the selectable marker gene. Due to the right-to-left orientation of T-DNA transfer (11), it is preferable to clone the gene of interest

next to the right border. Second, the backbone sequence of pSR8-30 relies on ampicillin resistance as a bacterial selectable marker gene, which is not useful in many *Agrobacterium* strains, and can compromise preferred treatments in many plant species for the subsequent elimination of *Agrobacterium* following co-cultivation of plant tissue. The pMOA1-5 series of minimal T-DNA vectors (12) overcomes these limitations, and also provides identical vectors with different selectable markers genes and a multiple cloning region with 12 unique restriction sites. However, our pMOA1-5 vectors are limited by poor plasmid yields, due to the pBIN19 backbone with a RK2 replicon that is unstable and has a low copy number (2,7). Furthermore, our vectors are sometimes limited by choice of restriction sites for the insertion of agriculturally useful genes and analysis of resulting transgenic plants. To overcome these limitations, we constructed a new series of minimal T-DNA binary vectors for *Agrobacterium*-mediated gene transfer.

All plasmid preparations, restriction digestions, and ligations were carried

Figure 1. The intermediary plasmids pMOA10-12. The plasmid pMOA10 was created by enzymatic restriction of the binary vector pSR8-30 (10) with *Hind*III and *Eco*RV to remove the *npt*II selectable marker gene and the transfer DNA (T-DNA) right border. A synthetic sequence encoding a *Sac*I site and a right T-DNA border flanked by *Hind*III and *Eco*RV sites replaced the deleted fragment to give pMOA10. A further synthetic sequence encoding multiple cloning sites (MCS) bound by *Hind*III and *Sac*I was then ligated into pMOA10 restricted with *Hind*III and *Sac*I to yield pMOA11. Following restriction of pMOA11 with *Cla*I and *Bam*HI, a 0.3-kb terminator region from the nopaline synthase gene (*nos*3') from pGEM5-preShiva-1 (gift from A. Gleave, HortResearch, Canterbury, NZ) was ligated as a *Cla*I-BamHI fragment to yield pMOA12. This latter cloning step also removed a *Bgl*III site and added another *Hind*III site between the *nos*3' and the *Cla*I site.

Figure 2. The complete binary vectors pMOA13 and pMOA33 and the transfer DNA (T-DNA) regions of pMOA14-17, pMOA30, and pMOA34-37. Different selectable marker genes were individually ligated as *Bam*HI-*Xba*I fragments, with a promoter region fused to a coding region from the pMOA1-5 series (12) into pMOA12 to give pMOA13-pMOA17. While this removes *Sal*I and *Hind*III sites from pMOA12, the ligation simultaneously recovers *Hind*III, *Sph*I, *Pst*I, and *Sal*I sites in their place. To provide a vector backbone with spectinomycin as selection in bacteria, the T-DNA of pMOA13 was isolated as a *Xho*I-*Eco*RI fragment, blunt ended, flanked by *Xho*I linkers, and ligated to the backbone of pART27 (5) isolated as a *Sal*I fragment to yield pMOA33. This effectively destroys both the *Sal*I and *Xho*I sites. The 1.7 kb nos-nptII-nos selectable marker gene conferring kanamycin resistance was then removed from pMOA33 by restriction with *Hind*III, which created pMOA30 that has no selectable marker gene, only a series of restriction enzyme sites. *Hind*III fragments containing four selectable marker genes from pMOA14-17 were then individually ligated into *Hind*III-restricted pMOA30, to give pMOA34-37. The selectable marker genes on the T-DNAs provide resistance to kanamycin (*NPT II*), hygromycin (*HPT*), methotrexate (*DHFR*), phosphinothricin (*BAR*), and bleomycin/phleomycin (*BLEO*), respectively.

out according to standard protocols for DNA cloning using *Escherichia coli* strain DH5 α as a host (13). Construction of the intermediary plasmids pMOA10-12 is described in Figure 1. To produce a series of minimal T-DNA binary vectors with different selectable marker genes, a promoter region fused to different coding regions were individually ligated into pMOA12 as *Bam*HI-*Xba*I fragments to give pMOA13-pMOA17 (Figure 2). This series of binary vectors retain the original pSR8-30 backbone, which is of limited value due to the

ampicillin-resistant bacterial marker gene (see above). Therefore, a more convenient vector backbone, which provides a resistance to spectinomycin in bacteria, was used to create pMOA30-37 (Figure 2). DNA sequencing verified the composition of the T-DNA regions of pMOA30, pMOA33, pMOA34, pMOA35, pMOA36, and pMOA37 (GenBank® accession nos. DQ869003, DQ869004, DQ869005, DQ869006, DQ869007, and DQ869008, respectively). We have repeatedly accomplished transfor-

mation of potato cultivar Iwa with the pMOA33-37 series using the protocols and concentrations of selective agents described for our pMOA1-5 series (12). In all cases the relative transformation efficiencies for the pMOA33-37 vectors were equivalent to our previous results with pMOA1-5 (12).

The simple binary vectors reported in this paper are based on the minimum features necessary for efficient plant transformation. pMOA30 provides the opportunity to create marker-free transgenic plants by cotransformation

experiments with any other binary vector. pMOA33-37 have very small T-DNAs (1660–2140 bp) with a different selectable marker gene tightly inserted between the left and right T-DNA borders. Between the marker gene and the right T-DNA border there is a short 85-bp multiple cloning site (MCS) for cloning genes of interest. The multiple cloning region includes two unique rare restriction sites to facilitate cloning options and analysis of T-DNA insertions in plant genomes. The high copy number *colE1* replicon on the backbone of pMOA33-37 means that sufficient plasmid can be extracted from 1.5 mL overnight cultures of *E. coli* for subsequent cloning manipulations. These vectors are specifically designed without any extraneous DNA fragments for the development of transgenic plants for commercial use in agriculture. The availability of a range of selectable markers genes is also important to allow transgene pyramiding by successive transformation. This is especially important in clonal crops where it is impossible to maintain the genetic integrity of an elite cultivar by combining traits through sexual hybridization (14).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. Hellens, R., P. Mullineaux, and H. Klee. 2000. A guide to *Agrobacterium* binary vectors. *Trends Plant Sci.* 5:446-451.
2. van Engelen, F.A., J.W. Molthoff, A.J. Conner, J.P. Nap, A. Pereira, and W.J. Stiekema. 1995. pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res.* 4:288-290.
3. Hellens, R.P., E.A. Edwards, N.R. Leyland, S. Bean, and P.M. Mullineaux. 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 42:819-832.
4. Becker, D., E. Kemper, J. Schell, and R. Masterson. 1992. New binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* 20:1195-1197.
5. Gleave, A.P. 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* 20:1203-1207.
6. Hajdukiewicz, P., Z. Svab, and P. Maliga. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* 25:989-994.
7. McBride, K.E. and K.R. Summerfelt. 1990. Improved binary vectors for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 14:269-276.
8. McCormac, A.C., M.C. Elliot, and D.-F. Chen. 1999. pBECKS2000: a novel plasmid series for the facile creation of complex binary vectors, which incorporates "clean-gene" facilities. *Mol. Gen. Genet.* 261:226-235.
9. Nap, J.P., P.L.J. Metz, M. Escaler, and A.J. Conner. 2003. The release of genetically modified crops into the environment: I. Overview of current status and regulations. *Plant J.* 33:1-18.
10. Düring, K. 1994. A plant transformation vector with a minimal T-DNA. *Transgenic Res.* 3:138-140.
11. Zambryski, P.C. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:465-490.
12. Barrell, P.J., Y.J. Shang, P.A. Cooper, and A.J. Conner. 2002. Alternative selectable markers for potato transformation using minimal T-DNA vectors. *Plant Cell Tissue Organ Cult.* 70:61-68.
13. Sambrook, J. and D.W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
14. Conner, A.J. and M.C. Christey. 1994. Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. *Biocontrol Sci. Technol.* 4:463-473.

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