

## Update section

Short communication

# A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome

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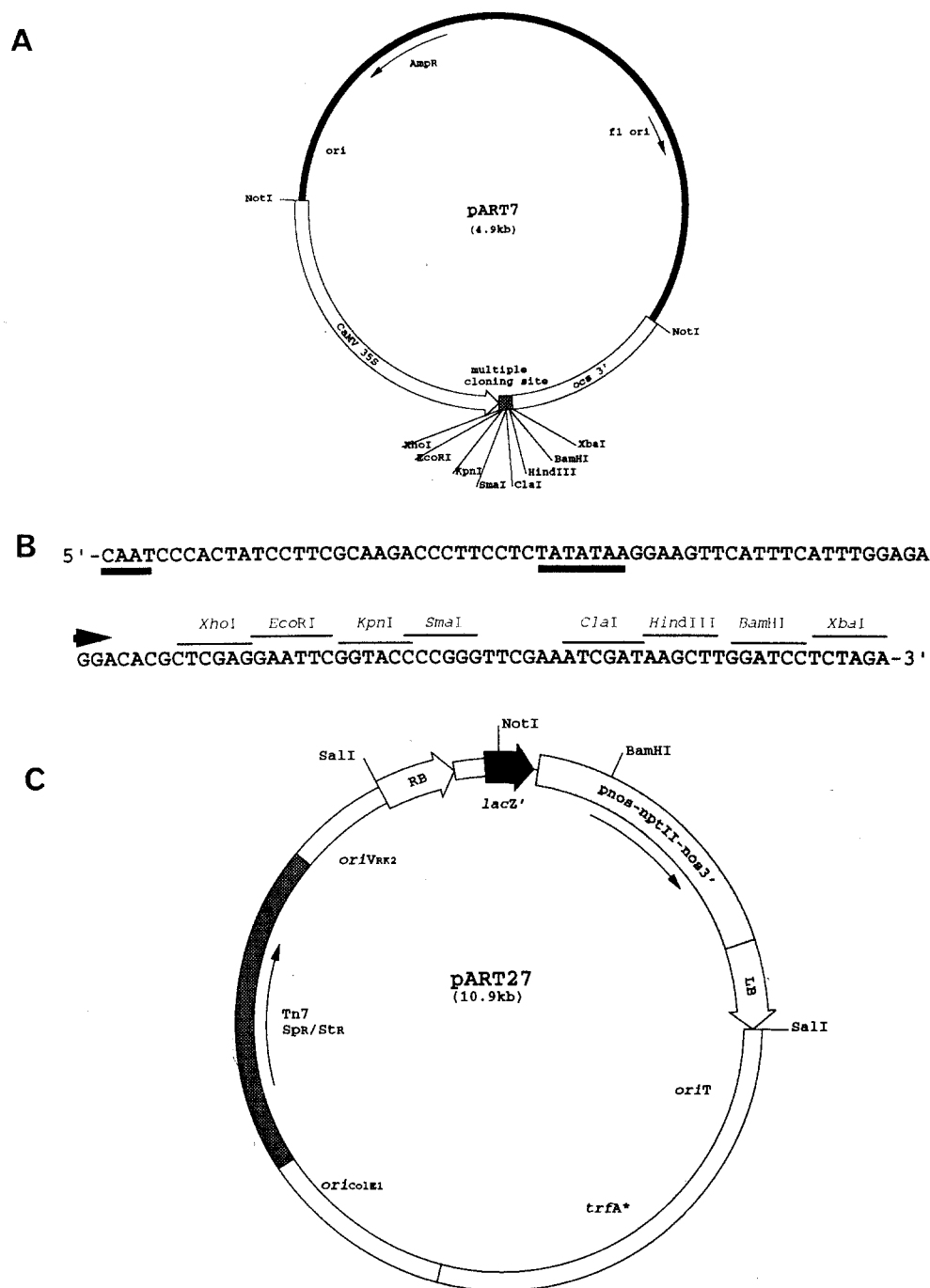
## Abstract

A versatile gene expression cartridge and binary vector system was constructed for use in *Agrobacterium*-mediated plant transformation. The expression cartridge of the primary cloning vector, pART7, comprises of cauliflower mosaic virus Cabb B-JI isolate 35S promoter, a multiple cloning site and the transcriptional termination region of the octopine synthase gene. The entire cartridge can be removed from pART7 as a *Not* I fragment and introduced directly into the binary vector, pART27, recombinants being selected by blue/white screening for  $\beta$ -galactosidase. pART27 carries the RK2 minimal replicon for maintenance in *Agrobacterium*, the *ColE1* origin of replication for high-copy maintenance in *Escherichia coli* and the Tn7 spectinomycin/streptomycin resistance gene as a bacterial selectable marker. The organisational structure of the T-DNA of pART27 has been constructed taking into account the right to left border, 5' to 3' model of T-DNA transfer. The T-DNA carries the chimaeric kanamycin resistance gene (nopaline synthase promoter-neomycin phosphotransferase-nopaline synthase terminator) distal to the right border relative to the *lacZ'* region. Utilisation of these vectors in *Agrobacterium*-mediated transformation of tobacco demonstrated efficient T-DNA transfer to the plant genome.

*Trans*-acting *vir* functions encoded by the tumour-inducing (Ti) or root-inducing (Ri) plasmids, and by the *Agrobacterium* chromosome [5, 8] act to promote the integration into the plant genome of DNA segments which are delimited by *cis*-acting T-DNA border sequences [2, 8]. These *trans*- and *cis*-acting features have led to the construction of a variety of plasmid vectors termed 'binary vectors' for use in *Agrobacterium*-mediated plant transformation [1, 11, 15]. This report describes

a versatile primary cloning vector and binary vector system for use in *Agrobacterium*-mediated plant transformation.

The expression cartridge of the primary cloning vector, pART7 (Fig. 1A), comprises the 35S promoter of a cauliflower mosaic virus (CaMV) isolate, CabbB-JI [4], and the octopine synthase gene (*ocs*) 3'-untranslated region [10], between which lies a multiple cloning site (*mcs*) offering 8 unique restriction sites (*Xho* I, *Eco* RI, *Kpn* I,



*Fig. 1.* A. Primary cloning vector, pART7. The pGEM9Zf<sup>-</sup>-derived backbone of the vector is represented by the dark line. The CaMV 35S promoter is represented by the open box with the arrow indicating the direction of transcription. The multiple cloning site is indicated and the *ocs3'* region is represented by the open box. B. Nucleotide sequence of the transcriptional elements of the CaMV 35S promoter and the multiple cloning site of pART7. The TATA box and CAAT box of the CaMV 35S promoter are underlined and the arrow represents the transcriptional start point [13]. The unique restriction sites of the multiple cloning site are indicated. C. Binary vector, pART27. The right border (RB) and left border (LB) are indicated by the arrowed boxes; the *lacZ'* region (encoding the *lac*  $\alpha$  peptide) is represented by the dark arrowed box and the chimeric *nptII* region is shaded (arrow

*Sma* I, *Hind* III, *Cla* I, *Bam* HI, *Xba* I) for cloning genes of interest downstream of the 35S promoter. This design permits the initiation of transcription at the promoter's natural mRNA start [13] and the absence of any AUG translational initiation signals in the mcs (Fig. 1B) ensures that translation will begin at the first AUG of the cloned DNA. The presence of the *ocs* 3'-untranslated region, which includes the polyadenylation signals [10] should act to terminate transcription. The entire expression cartridge (35-mcs-*ocs* 3') of pART7 is flanked by *Not* I sites facilitating its introduction into the binary vector, pART27 (described below). As *Not* I recognises the 8 bp sequence 5'-GCGGCCGC-3', of relative infrequency in most genomes, its occurrence in most genes of interest is rare. As the backbone of pART7 is derived from pGEM9Zf<sup>+</sup> standard mini-prep isolation procedures give high DNA yields from *E. coli* and the presence of the  $\phi$ 1 origin of replication, permits single-stranded DNA isolation of pART7 derivatives, useful for DNA sequencing and *in vitro* mutagenesis.

The backbone of the binary vector, pART27 (Fig. 1C), providing the RK2 minimal replicon [16] for replication in *E. coli* and *Agrobacterium*, the ColE1 replicon for elevated copy number in *E. coli* and the Tn7 spectinomycin/streptomycin resistance gene for bacterial selection, was derived from the binary vector pMON530 [15]. In designing the T-DNA of the binary vector the primary aim was to provide an organisational structure with a *lacZ'* region immediately 3' of the right T-DNA border and 'overdrive' enhancer element [14], followed by a chimaeric plant selectable marker and left T-DNA border, the latter reducing the frequency of random termination of T-strand transfer observed with vectors lacking such a border [7]. As T-strand transfer is thought to proceed from right to left border via a 5'–3' mechanism [17] then the *lacZ'* region and any DNA cloned into it (i.e. the expression cartridge and gene of interest) ought to be transferred

to the plant genome prior to the selectable marker and therefore a greater percentage of kanamycin-resistant plants are expected to contain the gene of interest. The right T-DNA border, 'overdrive' element, left T-DNA border and the chimaeric kanamycin resistance gene (nopaline synthase promoter-neomycin phosphotransferase-nopaline synthase terminator) of pART27 were derived from pGA643 [1]. A modified *lacZ'* region (*Sal* I-*Nde* I deletion) from pGEM5Zf<sup>+</sup> containing a unique *Not* I site to facilitate cloning of the expression cartridge of pART7, was introduced between the right T-DNA border and chimaeric kanamycin resistance gene and allows blue/white screening of pART27 recombinants. This T-DNA cartridge was introduced into the pMON530 derived replicon generating the binary vector pART27 (Fig. 1C).

pART27 can be selected for in both *E. coli* and *Agrobacterium* strains on media containing either spectinomycin (100  $\mu$ g/ml) or streptomycin (25  $\mu$ g/ml). The chimaeric *nptII* gene is also expressed in *E. coli* and *Agrobacterium* allowing kanamycin (20  $\mu$ g/ml) to be used as an alternative selectable marker. In contrast to binary vectors whose replicon is solely RK2-based, the presence of the ColE1 replicon in pART27 allows reliable mini-prep DNA isolation of binary vector derivatives for restriction analysis and direct transformation of *Agrobacterium*. In addition, pART27 derivatives can be introduced into *Agrobacterium* via matings as the vector also carries the RK2 derived origin of transfer (*oriT*) necessary for conjugal transfer [3]. pART27 also has an advantage over binary vectors based on the mini Ri plasmid replicon as it is compatible with both the resident Ti plasmids of *Agrobacterium tumefaciens* and the Ri plasmids of *Agrobacterium rhizogenes* allowing either host to be used for plant transformation.

Numerous binary vectors contain significant stretches of duplicated sequences as a consequence of driving transcription of both the gene

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denotes orientation of the coding region). The replication functions are indicated and the Sp<sup>R</sup>/St<sup>R</sup> bacterial selectable marker is represented by the dark box (arrow denotes orientation of coding region). Relevant restriction sites are shown, more detailed maps and information on vector construction are available upon request.

of interest and the plant selectable marker with copies of the same promoter. These duplicated sequences may be responsible for plasmid instability in either of the bacterial hosts and/or instability within the plant genome. During the construction of the vectors described here significant duplicated sequences were avoided to minimise the possibility of recombination events leading to deletions and/or rearrangements.

In order to test the functionality of the pART27 and the pART7 expression cartridge the *gus* reporter gene was introduced 3' of the CaMV 35S promoter followed by the introduction of the *Not* I CaMV 35S-*gus*-*ocs* 3' fragment into the *Not* I cloning site of pART27, generating pART278. *A. rhizogenes* A4T [12] harbouring pART27 or pART278 were used in standard leaf disc transformations of *Nicotiana plumbaginifolia*. Individual hairy root pieces generated from pART27 and pART278 transformation were stained for  $\beta$ -glucuronidase (GUS) activity in 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-glucopyranoside, 50 mM sodium phosphate buffer pH 7.0, at 37 °C. Roots derived from pART27 transformation showed no GUS activity, as was expected. Of 20 independent pART278-derived, kanamycin-resistant roots, 17 stained positive for GUS activity indicating the frequency of genuine transformants to be at least 85%. The GUS activity, as determined by the rate and intensity of blue colouration, varied considerably between root lines, probably as a consequence of gene copy number and/or positional effects. DNA extracted [9] from each of the 20 pART278-derived putative transformants was used as template in PCR reactions. The PCR screening with primers *gus*<sub>1</sub> and *gus*<sub>2</sub>, homologous to nucleotides 73–92 and 749–730 of the *gus* open reading frame [6], respectively, detected the presence of the 676 bp *gus* fragment in the 17 putative transformants which had stained positive for GUS activity, confirming the presence of the *gus* gene. PCR failed to detect the 676 bp *gus* fragment in the 3 kanamycin-resistant root lines which showed no detectable GUS activity, indicating these root lines were kanamycin-resistant 'escapes'. Probably as a consequence of the T-DNA organisational struc-

ture of pART27, 85% of the kanamycin-resistant roots generated by *A. rhizogenes*-mediated transformation of *N. plumbaginifolia* appeared to be genuine transformants based on both histochemical analysis and PCR detection.

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