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pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation

Roger P. Hellens*, E. Anne Edwards, Nicola R. Leyland, Samantha Bean and Philip M. Mullineaux

*John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK (*author for correspondence; e-mail: hellens@bbsrc.ac.uk; fax: +44 1603 456844)*

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Abstract

Binary Ti vectors are the plasmid vectors of choice in *Agrobacterium*-mediated plant transformation protocols. The pGreen series of binary Ti vectors are configured for ease-of-use and to meet the demands of a wide range of transformation procedures for many plant species. This plasmid system allows any arrangement of selectable marker and reporter gene at the right and left T-DNA borders without compromising the choice of restriction sites for cloning, since the pGreen cloning sites are based on the well-known pBluescript general vector plasmids. Its size and copy number in *Escherichia coli* offers increased efficiencies in routine *in vitro* recombination procedures. pGreen can replicate in *Agrobacterium* only if another plasmid, pSoup, is co-resident in the same strain. pSoup provides replication functions *in trans* for pGreen. The removal of *RepA* and *Mob* functions has enabled the size of pGreen to be kept to a minimum. Versions of pGreen have been used to transform several plant species with the same efficiencies as other binary Ti vectors. Information on the pGreen plasmid system is supplemented by an Internet site (http://www.pgreen.ac.uk) through which comprehensive information, protocols, order forms and lists of different pGreen marker gene permutations can be found.

Introduction

Stable plant transformation is commonly achieved by *Agrobacterium tumefaciens*-mediated procedures (Ellis, 1993). *Agrobacterium* is a plant pathogen which causes the formation of crown galls or tumours in tissues infected by the bacterium (see Sheng and Citovsky, 1996, and Gheysen *et al.*, 1998, for a detailed review). Briefly, the determinants for establishing and sustaining tumours are located mostly on large (>200 kb) Ti (tumour-inducing) plasmids. The T-DNA and the virulence (*vir*) region are two distinct regions of all Ti plasmids which are essential for *Agrobacterium*-mediated plant transformation. The T-DNA is a discrete section of the Ti plasmid bounded by 25 bp imperfect repeats termed the right (RB) and left borders (LB). The T-DNA is transferred to, and integrated in, the host cell nuclear genome at the onset of infection. The processing of the T-DNA and its transfer to the host plant cell nucleus is achieved primarily by the concerted action of about 20 *vir* gene products. All the plasmid-encoded *vir* genes reside in a region of the Ti plasmid. Ti plasmid-encoded *vir* genes can function *in trans* to promote the transfer of T-DNAs from co-resident plasmids to recipient plant cells (Hoekema *et al.*, 1983). Such T-DNA-containing plasmids are termed Ti vectors (Bevan, 1984; Guerineau and Mullineaux, 1993). Genes and sequences to be transformed into plants are inserted between the LB and RB of the Ti vector T-DNA.

Binary Ti vectors are able to replicate in *Escherichia coli* and *Agrobacterium* species. In such plasmids, a broad-host-range replication locus is often

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ007829 (pGreen0000).

used, such as that from pRK2, which functions in a wide range of gram-negative bacteria (Doran *et al.*, 1998). The pRK2 replication origin is used in pBIN19 and derived Ti vectors (Bevan, 1984), or has been modified to separate the replication origin from the *trans*-acting RK2-specific replicase gene (Koncz and Schell, 1986) where the replicase gene (*trfA*) has been integrated into the *Agrobacterium* genome. Alternatively, binary Ti vectors can harbour two replication origins (*oris*), one for use in *E. coli* (e.g. from pColE1) and one for *Agrobacterium* (e.g. from a pRi), as in the pCGN series of plasmids (McBride and Summerfelt, 1990).

The T-DNA region of early binary vectors was subcloned from wild-type Ti plasmids such as pTiT37, like in pBIN19 (Bevan, 1984). More recently, synthetic LB and RB fragments have been derived from the 25 bp repeat structure (McCormac *et al.*, 1997). Between these border sequences there is often a selectable marker gene, coding for antibiotic or herbicide resistance, configured for expression in and required for the selection of transformed plants. All the binary Ti vectors contain an antibiotic resistance gene, suitable for selection in both *E. coli* and *Agrobacterium*. In the more versatile plasmids there is also a series of unique restriction sites within a β-galactosidase gene (*lacZ*′) to allow complementation-based screening for recombinant plasmids (Norrander *et al.*, 1983).

We set out to design a new binary Ti vector system that would address the main drawbacks of current plasmids. The efficiency of *in vitro* recombination procedures is inversely proportional to the size of the plasmid DNA (Sambrook *et al.*, 1989). With an increased requirement for the transfer of large pieces of DNA into plants (Simeons *et al.*, 1986; Olszewski *et al.*, 1988; Hamilton *et al.*, 1996), the size of binary Ti vector should be kept to a minimum. Several selectable marker and reporter genes are used in different transformation protocols (Croy, 1993) but these are rarely interchangeable between vectors. Different selectable marker genes may need adapting to the requirements of individual transformation protocols or to specific experiments such as those using multiple resistance genes (Battacharyya *et al.*, 1994). Such requirements should not compromise the flexibility of these plasmids as cloning vectors through loss of convenient restriction sites. Plasmid manipulations are also easier if these vectors replicate in *E. coli* to high copy number. Our new binary Ti vector, pGreen, addresses these issues by reduced plasmid size, transformation selection flexibility and an extensive multiple cloning site. In addition, this vector provides additional flexibility for further development of *Agrobacterium* transformation technology. The pGreen plasmid system is complemented by an Internet site: www.pGreen.ac.uk

Materials and methods

Materials and in vitro *cloning procedures*

All *in vitro* recombination techniques employed were standard (Sambrook *et al.*, 1989). Site-directed mutagenesis was based on the pAlter system (Promega, Wisconsin) as recommended by the manufacturer. Sequencing used a dye terminator labelling procedure and an ABI 373A Automated Sequencing machine according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The sequence of pGreen is available in the EMBL/GenBank database (AJ007829) and on the pGreen website.

All restriction and DNA modifying enzymes were from Boehringer Mannheim (Lewes, UK). All antibiotics were purchased from Sigma (St. Louis, MO).

Transformation of bacteria

E. coli (unless otherwise mentioned, strain DH5α) was transformed routinely by the procedure of Hanahan (1983). Electroporation-competent *Agrobacterium tumefaciens* (LBA44404, Hoekema *et al.*, 1983; GV2260, Deblaere *et al.*, 1985; GV3280, Zambryski *et al.*, 1983; EHA105, Hood *et al.*, 1993; AGL-1, Lazo *et al.*, 1991), and *A. rhizogenes* LBA9402 (Ooms *et al.*, 1985) cells were prepared according to Shen and Forde (1989) and electroporated in 2 mm cuvettes (BioRad, Hemel Hempstead, UK), with the following conditions: 2.5 kV, 400 Ω, 25 μF and 10–100 ng plasmid DNA. The electroporated cells were recovered in Luria broth for 4 h at 28 °C and plated on Luria broth agar plates (Sambrook *et al.*, 1989) containing kanamycin sulfate (50 μg/ml) and rifampicin (100 μg/ml). Alternatively, plasmids were transformed into *Agrobacterium tumefaciens* using a freeze-thaw method (An, 1988).

Construction of the pGreen plasmids

The components of pGreen were constructed as follows.

The pSa replicon and construction of pSoup. A 1.7 kb *Sau*3A partial fragment of pGV1122

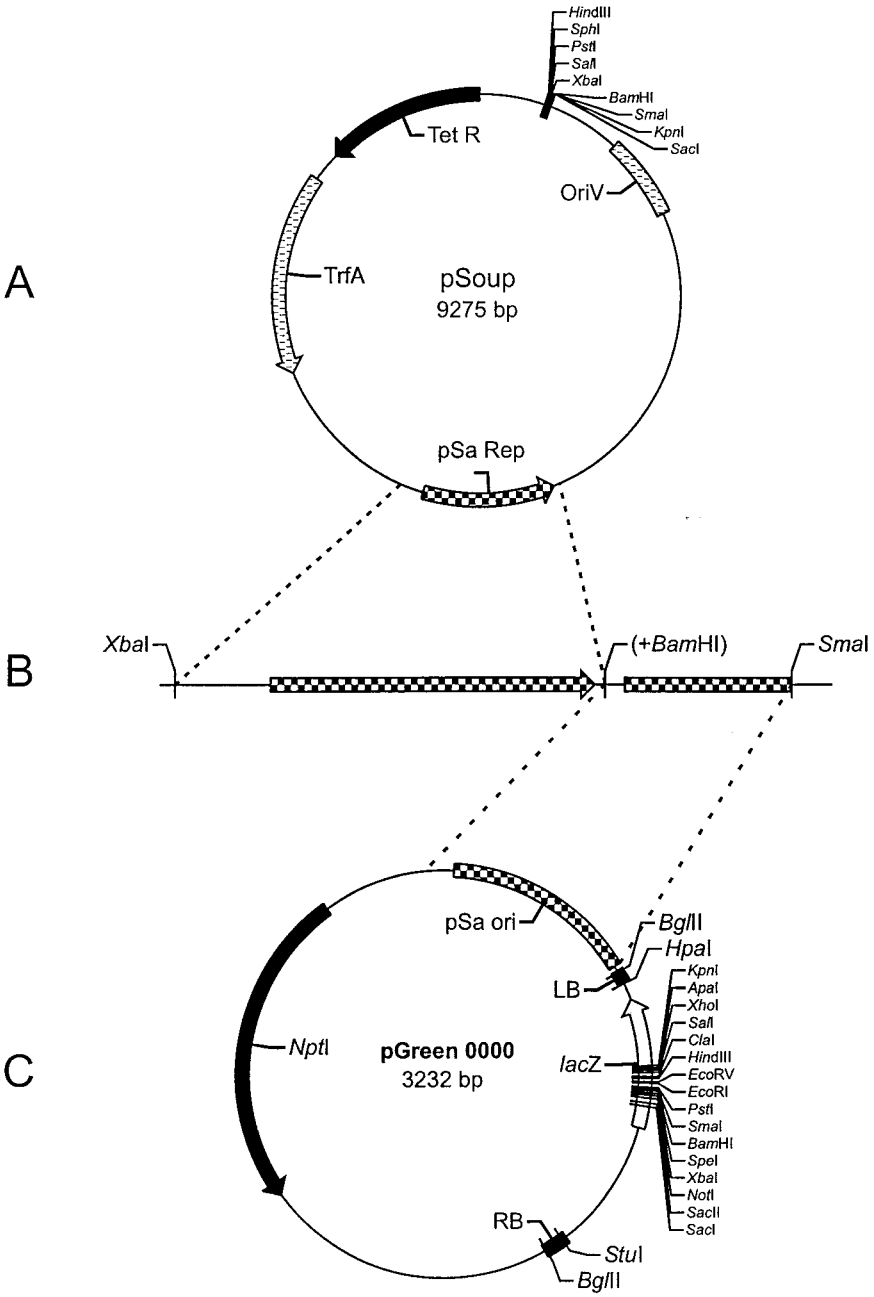

Figure 1. pSoup (A), a modified RK2 plasmid which carried the pSa replicase gene, and pGreen (C), the T-DNA cloning vector with pSa replication origin. B. The pSa replication locus (Chang and Cohen, 1978) was modified by site-directed mutagenesis (SDM) to introduce a *Bam*HI site, allowing these two regions to be separated.

(ATCC37171) was cloned into the *Bam*HI site of pJIT134 (Guerineau and Mullineaux, 1993) and selected for replication in *A. tumefaciens* LBA4404. The 1.7 kb *Sau*3A fragment in the resulting plasmid, called pJIT134Sa, was sequenced. Site-directed mutagenesis (Figure 1B) introduced a *Bam*HI site (mutagenic oligo 5′-CTTGGTCAAGGATCCCCATCCAAC-3′) between the pSa-*RepA* and pSa-*ori*, creating pJIT134SaBam. The 1.2 kb *Xba*I-*Bam*HI fragment containing the *RepA* gene was cloned between the *Bgl*II sites of pBIN19 (Bevan, 1984; Frisch *et al*., 1995), replacing the pBIN19 T-DNA. The bacterial kanamycin selection gene, *NptIII*, of this pBIN19 derivative was replaced by inserting an *Eco*RI-*Sty*I fragment, harbouring the tetracycline resistance gene, from pAlter (Promega) between the remaining pBIN19 *Eco*RV sites to produce pSoup (Figure 1A).

pGreen backbone. The kanamycin resistance gene (*NptI*) from pACYC177 (Chang and Cohen, 1978) was cloned as a *Nhe*I-*Nco*I fragment into the *Spe*I-*Bsp*HI sites of pBluescript SKII+, creating intermediate I. The *Nco*I site was introduced and restriction sites, which would have been duplicated in pGreen polylinker, were removed by site-directed mutagenesis (mutagenic oligos: *Xho*I (5′-CGTCTTGCTCAAGGCCGCGAT-3′), *Cla*I (5′-CGACAATCTACCGATTGTATG-3′), *Sma*I (5′-CTG CGATCCCAGGGAAAACAG-3′), *Hin*dIII (5′-AAAT GCATAAAGTTTTGCCAT-3′) and *Nco*I (5′-TGGTT-GTAACCATGGCAGAGCA-3′); (the underlined letters are the modified restriction recognition sequences). Two complementary oligos (5′-GAATTCA-GATCTA-3′ and 5′-ACATGTAGATCTG-3′ respectively) were annealed and inserted between the *Eco*RI and *Afl*III sites, to introduce a unique *Bgl*II site, this was intermediate II. The pSa-*ori* sequence was inserted as a *Bam*HI-*Sma*I fragment from pJIT134Sa-Bam into the *Bam*HI-*Sma*I sites of intermediate II (remaining from the original pBluescript plasmid). These sites along with intervening *Pst*I and *Not*I sites were removed by successive rounds of treatment with T4 DNA polymerase I (T4 polI) and religation. The *Stu*I site in the *NptI* promoter and *Cla*I sites, introduced when the pSa-*ori* was inserted, were removed by transformation into *E. coli* strain SCS110 (*dam;dcm*), digestion with T4 polI and re-ligation. This produced the pGreen backbone which was ready to receive the T-DNA cassette.

The T-DNA. The complementary oligonucleotides (5′-CATGAAGGCCT**TGACAGGATATATTGGCG GGTAAAC**TAAGTCGCTGTATGTGTTTGTTTGAG ATCT-3′ and 5′-CATGAGATCTCAAACAAACACAT ACAGCGACTTAGTTTACCCGCCAATATATCCTGT CAAGGCCTT-3′) were annealed to produce a DNA fragment consisting of a *Stu*I site (underlined), the RB sequence (in bold), the RB 'overdrive' sequence (in italics) and a *Bgl*II site (underlined). This RB DNA fragment was inserted into the *Afl*III site of pBluescript SKII− and its orientation determined by sequencing. A recombinant plasmid (intermediate A) was selected which had the orientation of the RB fragment such that the *Stu*I site was nearest to the SK multiple cloning site. Two further oligonucleotides (5′-TCCACACATTATACGAGCCGATGATTAATTGTC AACAGATCT**TGGCAGGATATATTGTGGTGTA AAC**GTTAAC-3′ and 5′-GGTAACGTTTACACCAC AATATATCCTGCCAAGATCTGTTGACAATTAAT CATCGGCTCGTATAATGTGTGGA-3′) were then annealed to produce an LB DNA fragment consisting of *Hpa*I and *Bgl*II site (underlined) and LB sequence (in bold). This fragment was inserted between the two *Ssp*I sites of intermediate A, simultaneously deleting the pBluescript SKII− f1 *ori*. This 815 bp *Bgl*II fragment was cloned into the pGreen backbone to produce pGreen0000 (Figure 1C) and pGreen1000 (corresponding to RB-LB and LB-RB orientations). This strategy was also followed with pBluescript KSII−, to produce the T-DNA for pGreen 3000 and pGreen4000.

Construction of selectable marker and reporter gene cassettes

The promoter-terminator cassettes were constructed for the insertion of marker gene sequences. The 35S CaMV promoter-19S CaMV terminator cassette was based on pJIT61, equivalent to pJIT60 (Guerineau and Mullineaux, 1993) except that pJIT61 contains a single CaMV 35S enhancer region and multiple-cloning-site (from CaMV 35S promoter) *Hin*dIII, *Xba*I, *Bam*HI, *Sma*I, *Sac*I and *Eco*RI restriction (to CaMV 19S terminator). The *Kpn*I site at the 5′ end of the 35S promoter was converted to an *Eco*RV site by digestion and religating with the self-complementary oligonucleotide (5′-GATATCGTAC-3′). In the same way the *Bgl*II site at the 3′ end of the 19S CaMV terminator sequence was converted to an *Eco*RV site using the self-complementary oligonucleotide (5′-GATCGATATC-3′) to produce the 35S cassette (Figure 2). The plasmid pSLJ261 (Jones *et al.*, 1992)

Table 1. Mutagenic oligonucleotides used in site-directed mutagenesis for a range of marker genes. kan, kanamycin resistance encoded by *aph*3′II/*NptII neo*, the neomycin phosphotransferase II gene (Bevan, 1984; Yenofsky *et al.*, 1990); bar, bialaphos resistance from *S. hygroscopicus* encoded by *pat*, the phosphinothricin acetyl transferase gene (De Block *et al.*, 1987); sul, sulfadiazine resistance (Guerineau *et al.*, 1990); GUS, β-glucuronidase, encoded by the *uidA* gene (Jefferson *et al.*, 1987); GFP, green fluorescent protein (Haseloff *et al.*, 1997). The original restriction site sequence is underlined and the affected codon separated by spaces. The modified base is double-underlined. Also indicated is the base modification and corresponding amino acid conservation for each restriction site.

gene	internal site	modifying oligonucleotide
kan	*Nco*I	TCGTGACC CAC GGCGATGCCT T⇨C His
hyg	*Sac*II	CAGGATCG CCA CGC CTCCGGGCG G⇨A Pro G⇨C Agr
	*PstI+Nco*I	CCGCTGTT CTT CAGCCGGTCGCGGAG GCT ATGGATGCG G⇨T Leu C⇨T Ala
	*Eco*RI	CATGGG GAG TTCAGCGAGA A⇨G Glu
bar	*Apa*I	CCTACGCG GGT CCCTGGAAG C⇨T Gly
	*Sal*I	CGGCCGAG TCA ACCGTGTAC G⇨A Ser
	*Sac*II	ATGCCCCC CGT GGCATGCTG G⇨T Arg
	*Kpn*I	GCCTGCCG GTG CCGCCCCGTC A⇨G Val
	*Bgl*II	CCGTCACC GAA ATCTGATGAC G⇨A Glu
sul	*Sac*II	CTGTCACC GCT GCGATCGAAA G⇨T Ala
	*Stu*I	ACGCGAGG CCA GTATCGCCGG T⇨A Pro
	*Eco*RV	ACCTGAAC GAC ATCCAAGGAT T⇨C Aps
	*Pst*I	AGGCGGAC TGT AGGCTGGTGG C⇨T Cys
	*Bgl*II	CGCCTGGA GAC CTGCGAAGCG T⇨C Asp
GUS	*Eco*RV	GGGTGGAC GAC ATCACCGTGG T⇨C Asp
	*Eco*RV	CAGAGTGT GAC ATCTACCCGC T⇨C Asp
GFP	*Nco*I	TACCTGTT CCT TGGCCAACAC A⇨T Pro
	*Hpa*I	TCAAAGTT AAT TTCAAAATTA C⇨T Tyr

was the starting plasmid for the construction of the nos promoter-terminator cassette. The internal GUS gene of this plasmid was replaced with a *Xho*I-*Xba*I fragment from the pBluescript multiple cloning site. The GUS-deleted pSLJ261 was prepared from the *E. coli dam*− strain GM2109 and digested with *Bcl*I, which cuts 5′ to the *nos* promoter, and converted to an *Eco*RV site by digestion and re-ligation with the self-complementary oligonucleotide (5′-GATCGATATC-3′). In the same way a *Hin*dIII site, 3′ of the *nos* terminator, was converted to a second *Eco*RV site with

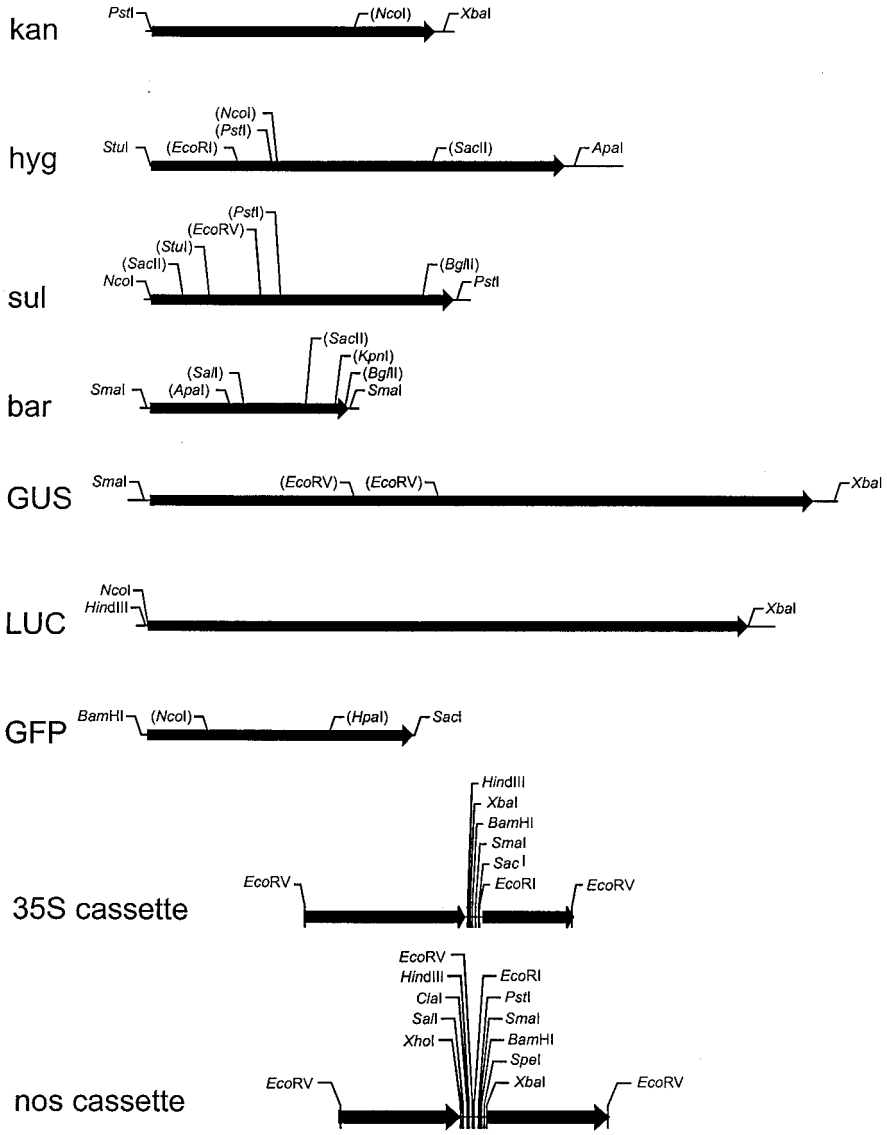

Figure 2. The reporter and selectable marker genes used for insertion into the *nos* (637 bp) and CaMV 35S (678 bp) cassettes. The restriction sites in parenthesis were removed by SDM. A list of the mutagenic oligonucleotides is available from the authors on request. The genes are *kan* (kanamycin resistance, 869 bp), *hyg* (hygromycin resistance, 1188 bp), *sul* (sulfadiazine resistance, 912 bp), *bar* (bialaphos resistance, 615 bp), GUS (*uidA*; β-glucuronidase, 1940 bp), LUC (firefly luciferase, 1765 bp) and GFP (green fluorescent protein, 745 bp). Also indicated are the restriction sites flanking each of the genes, along with all other restriction sites are deleted in the 35S and *nos* cassettes.

the oligonucleotide (5′-AGCTGATATC-3′) to produce the *nos* cassette (Figure 2).

Both these basic promoter-terminator cassettes were precursors for all of the CaMV 35S and *nos* marker gene cassettes (Figure 2). Restriction sites in the cassettes which would have been duplicated in the pGreen cloning sites were removed by site-directed mutagenesis. Four selectable marker genes – *kan* (*aph*3′II/*Npt*II; kanamycin resistance, Bevan *et al.*, 1983) from pJIT134 (Guerineau and Mullineaux, 1993), *hyg* (*aph*IV/*Npt*IV; hygromycin resistance, Waldron *et al.*, 1985) from pJIT6 (Guerineau and Mullineaux, 1993), *sul* (*sul*I; sulfadiazine resistance, Guerineau and Mullineaux, 1993) from pJIT92 (Guerineau *et al.*, 1990) fused to Rubisco chloroplast transit peptide from pJIT117 (Guerineau *et al.*, 1989) and *bar* (*pat*; bialaphos resistance, De Block *et al.*, 1987) from pJIT82 (Guerineau and Mullineaux, 1993) – and two

Table 2. Nomenclature of pGreen derivatives used in this work. The numbers are derived from the full list of all permutations of all marker and reporter genes which is available on the pGreen Website. (+) and (−) refer to outward and inward direction of transcription of the genes relative to the pGreen0000 T-DNA (Figure 1).

Plasmid	Selectable marker and reporter genes	
	LB	RB
pGreen0000	–	–
pGreen0029	*nos-kan* (+)	–
pGreen0049	*nos-kan* (+)	35S LUC (−)
pGreen0229	*nos-bar* (+)	–

Table 3. Segregation data for the seed of: self-crossed primary transgenic tobacco plants transformed with pGreen0029, back-crossed petunia plants transformed with a modified pGreen0029 and self-crossed pea transformed with a modified version of pGreen 0229.

Transgenic tobacco	
kan-resistant	kan-sensitive
38	11
39	10
29	20
47	2
39	5
39	8
48	1
41	8
49	1
38	10
38	11
41	9
Transgenic petunia	
T-DNA	no T-DNA
11	9
19	22
5	7
7	5
Transgenic pea	
bar-resistant	bar-sensitive
2	1
2	1
14	3

reporter genes – GUS (*uid*A; β-glucuronidase; Jefferson *et al.*, 1987) from pJIT166 (Guerineau *et al.*, 1992) and GFP (green fluorescent protein) from mGFP-4 (Haseloff *et al.*, 1997) – have been modified. The mutagenic oligonucleotides (Table 1) were designed such that the amino acid sequence was unchanged. In addition, a synthetic firefly luciferase gene (LUC+), with internal restriction sites already removed, which is available from Promega (Lonsdale *et al.*, 1998), was cloned into 35S and *nos* cassettes. Intervening restriction sites were removed after insertion of coding sequences into promoter/terminator cassettes by using T4 DNA polI treatment of DNA cut with the restriction enzyme, followed by re-ligation and selection for loss of the site (Figure 2).

Analysis of plasmid stability

Single colonies of *Agrobacterium* (LBA4404) harbouring pGreen0029 (Table 2), on LB Agar and antibiotic selection, were used to inoculate 10 ml of LB with appropriate antibiotic, and incubated at 30 °C with shaking. Of this overnight culture 100 μl was used to re-inoculate 10 ml of LB in the absence of antibiotic selection; at the same time a serial dilution of the bacteria was plated onto selective and non-selective media. The total viable count and antibiotic-resistant bacterial count was used to calculated the mean colony-forming units per ml of culture, using the data from the dilution series. The inheritance of plasmid was monitored for 7 overnight incubations.

Plant transformation

Protocols for plant transformation were as follows. *Arabidopsis thaliana* (ecotype Colombia) was transformed by the root explant method (Valvekens *et al.*, 1989) and the vacuum infiltration method (Bechtold, 1989). Cabbage was transformed as previously described for oil-seed rape (Moloney *et al.*, 1989). Transformation of pea (Bean *et al.*, 1997), potato (Edwards *et al.*, 1995), petunia (Horsch *et al.*, 1985) and tobacco (Guerineau *et al.*, 1989) was as previously described. Preparation of plant DNA and associated Southern blotting techniques were employed as described in the quoted papers for these particular species. ^{32}P-labelled probes were prepared from DNA fragments by a random priming method (Feinberg and Vogelstein, 1983).

Results

Development of the pGreen vector and the dual plasmid system

pSoup

For replication in *Agrobacterium*, the pSa replication locus has been used in the pGreen vector. This locus is defined by a 1.7 kb *Sau*3A fragment from the plasmid pGV1122 (Okumura and Kado, 1992). Sequencing of this fragment showed that the pSa replication locus consists of an *ori* and *RepA* gene (Figure 1B). Pilot experiments established that the pSa-*RepA* gene can act *in trans* on the *ori*, in a manner similar to that described for the RK2 replication and replication-origin elements (Koncz and Shell, 1986), and was not needed on the pGreen vector, offering a substantial saving on the size of the binary vector. Therefore, pSa-*RepA* was subcloned into pBIN19, replacing the T-DNA region. The pBIN19 *NptIII* gene, coding for bacterial resistance to kanamycin, was replaced with the tetracycline resistance gene (*tet*) from pAlter (Promega), to create pSoup (Figure 1A). This plasmid has the pRK2 replication origin of the compatibility group incP1 (Thomas *et al.*, 1985) whereas the pSa origin is in the IncR compatibility group (Okumura and Kado, 1986), and as such these two plasmids can co-reside in *Agrobacterium*.

pGreen

The pGreen vector (Figure 1C) is based on the general-purpose cloning vector, pBluescript (Alting-Mees and Short, 1989) and therefore contains a pColE1 *ori* for replication in *E. coli*. This plasmid's ampicillin resistance gene was replaced with the *NptI* gene (encoding kanamycin resistance) from pACYC 177 (Chang and Cohen, 1978), the pSa-*ori* was inserted, the f1 *ori* and *Lac Z′* region deleted and a *Bgl*II site was left for the introduction of a T-DNA cassette (see Materials and methods).

A T-DNA cassette consisting of a 813 bp *Bgl*II fragment, containing the pBluescript SKII *LacZ′* gene and multiple cloning sites, flanked by synthetic LB and RB sequences, derived from the border sequences of pTiT37, and with an additional T-DNA transfer enhancer ('overdrive') motif immediately adjacent and external to the RB (Slightom *et al*., 1985; van Haaren *et al.*, 1988), was inserted to create pGreen0000 (Figure 1C).

Selectable marker and reporter gene cassettes

The basic pGreen vectors contain no selectable marker or reporter genes for plant transformation. Immediately internal to the T-DNA LB and RB are unique *Hpa*I and *Stu*I sites for the insertion of selectable marker or reporter genes fused to promoters and flanked by *Eco*RV sites (Figures 1 and 2). Four selectable marker genes, *kan*, *hyg*, *sul* and *bar*, and two reporter genes, GUS and GFP, have been modified, by site-directed mutagenesis, to remove all the restriction sites which would have been duplicated in the pGreen multiple cloning site. The *kan* gene (*NptII*) does not contain the mutation that can affect its function as a selectable marker gene in some plant species (Yenofsky *et al.*, 1990). The amino acid sequence of all these enzymes was not affected by the DNA sequence changes introduced (Table 1) and, where possible, the codon usage was modified to those favoured by plants (Murray *et al.*, 1988). In addition, a synthetic firefly luciferase gene (LUC+) available from Promega (Lonsdale *et al.*, 1998) also fulfilled the above criteria. These genes were cloned between the CaMV 35S promoter and the CaMV 19S terminator and between the nopaline synthase (*nos*) promoter and the *nos* terminator (Figure 2) and all extraneous sites at cloning junctions were removed.

pGreen nomenclature

We have assigned a number from 1 to 728 to all the possible 35S- and *nos*-containing cassettes cloned into the LB and/or RB cloning sites of pGreen0000/1000 (see Materials and methods). A KS version of pGreen, where the polylinker is in the opposite orientation with respect to the LB and RB, is available, though not extensively tested, and is called pGreen3000/4000. This designation of marker gene combinations can be assigned to any pGreen cassette variant produced in the future. A complete list of the different combinations is available on the pGreen Internet site (see below) and the details of cassettes described in this paper are in Table 2.

Plasmid handling and copy number in Agrobacterium

In *Agrobacterium* species the pGreen plasmid requires the function of the *RepA* gene from pSoup (Figure 1) to successfully replicate. pGreen contains no mobilisation function (which permitted further savings in size) and so the plasmid is introduced into

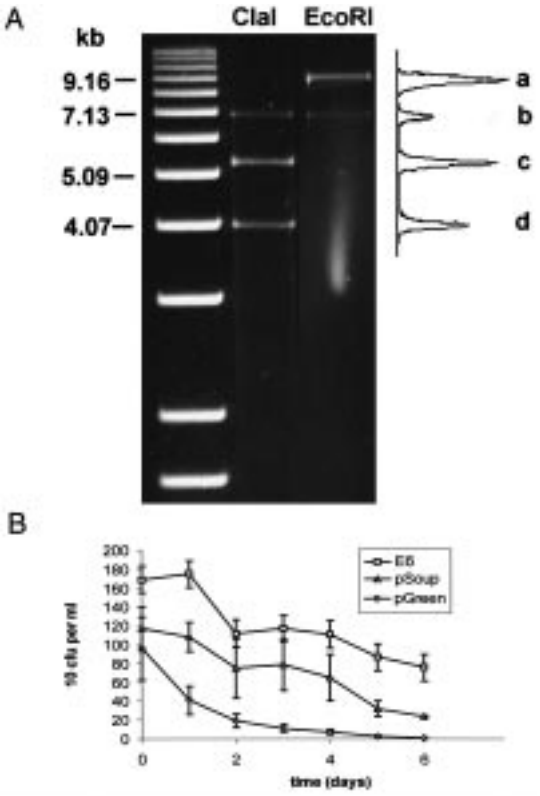

Figure 3. A. Plasmid DNA isolated from *Agrobacterium tumefaciens* strain LBA4404 harbouring pSoup and pGreen0049 (Table 2). The plasmid DNA was prepared by an alkaline lysis procedure and digested with either *Cla*I or*Eco*RI, and separated by electrophoresis through a 20 cm Tris-acetate-EDTA (TAE) 0.8% w/v agarose gel at 1.75 V/cm for 16 h. Densitometry of the ethidium bromide-stained gel image was carried out with a GelWorks 1D Advanced v 3.0 imaging system (BioRad, Hemel Hempstead, UK). Band a is *Eco*RI-linearised pSoup, band b is linear pGreen0049 generated with either *Eco*RI or *Cla*I and bands c and d are *Cla*I fragments of pSoup. B. Plasmid stability of pE6.

Agrobacterium using either electroporation or freeze-thawing (see Materials and methods). The pGreen and pSoup plasmids (Figure 1) can be used in a mixed electroporation. In this instance, selection for co-transformed *Agrobacterium* can be achieved using kanamycin-containing medium only, since pGreen cannot replicate in *Agrobacterium* without pSoup being co-resident. Alternatively, electro-competent *Agrobacterium* containing the pSoup can be generated, by selection for tetracycline resistance, and subsequently re-electroporated with pGreen and selection for kanamycin-resistant colonies: *A. tumefaciens* strains LBA4404, GV2260, GV3280, AGL-1 and EHA105 and *A. rhizogenes* strain LBA9402 support pGreen/pSoup replication.

An overnight culture of *Agrobacterium* (LBA4404) containing pGreen0049 (Table 1) and pSoup were used to estimate the relative copy number of each plasmid. *Agrobacterium* incubated overnight in Luria broth (Sambrook *et al.*, 1989) with 50 μg per ml kanamycin at 30 °C achieved an OD (650 nm) of 0.6±0.1 and was still in logarithmic growth. Plasmid DNA was extracted from these bacteria, and digested with restriction enzymes, and separated on agarose gels. The fluorescence of intercalated ethidium bromide into linear DNA is proportional to the DNA size, and this was used to estimate the relative weight of pGreen to pSoup DNA of the *Agrobacterium* in the culture. Figure 3A shows the ethidium stain for two restriction enzyme patterns: *Cla*I and *Eco*RI. pSoup was linearised by *Eco*RI to give a band of 9.0 kb (fragment a, Figure 3A), and cut twice by *Cla*I to 5.0 kb and 4.0 kb (fragments c and d, Figure 3A). pGreen0049 was linearised to 6.9 kb by both enzymes (fragment b, Figure 3A). The fluorescent profile for these ethidium-stained gels was analysed by GelWorks 1D (UVP UK) and indicated the linear relationship of fluorescence to electrophoresis mobility of fragments a, c and d, corresponding to a regression coefficient relating fluorescence to DNA size (fluorescence $= 1.42\times$ fragment size $- 7629$; $R^2 = 0.94$). Based on these calculations the expected fluorescence for a DNA fragment the size of fragment b and in the same molar ratio as fragments a, c and d was estimated at 17 569. The actual fluorescence for fragment b (4751±193.6) was used to estimate the relative copy number of pGreen to pSoup as ca. 3:11. Data for 3 restriction enzymes of 3 plasmid DNA preparations from independent *Agrobacterium* cultures were used in this estimation.

The stability of pGreen was measured by removing the plasmid selection from liquid media, and assaying the proportion of viable colonies which contained antibiotic resistance over 6 subsequent days. E6, a pBIN19-derived plasmid, generated by replacing the pBIN19 T-DNA contained within a *Bgl*II fragment with the 813 bp T-DNA-containing the *Bgl*II fragment of pGreen, was used as a control plasmid construct. Two bacterial strains, LBA4404::E6 and LBA4404::pGreen0000;pSoup, were grown as described in Materials and methods. E6- and pGreen-containing bacteria were identified by kanamycin resistance, while pSoup-containing bacteria were identified by resistance to tetracycline. The proportion of resistant colonies was assayed for 6 overnight incubations and is displayed in Figure 2B. Both the kanamycin-resistant E6 and the tetracycline-resistant

pSoup appear to be slightly unstable in *Agrobacterium*: the number of bacteria, after overnight incubation, retaining the E6 and pSoup plasmids dropped to 50% after 5.5 days and 4.5 days respectively. Both these plasmids contain the RK2 replication origin. The kanamycin-resistant pGreen plasmid is less stable in *Agrobacterium*, and after only 1 day, 50% of *Agrobacterium* colonies contained the pGreen plasmid.

Plant transformation

A range of experimental and crop plant species have been transformed with pGreen: *Arabidopsis*, pea, tobacco, petunia, tomato, potato and cabbage. Figure 4 shows the molecular analysis for transformants of four of these plant species.

An intron-containing CaMV 35S-GUS gene (R.P. Hellens, unpublished data) was cloned into pGreen0229 (Table 2) and used in *Agrobacterium*-mediated transformartion of *Pisum sativum* cv. Puget (Bean *et al.* 1997). Seven bialaphos-resistant transformed plants were recovered from 200 seed embryos, corresponding to a transformation efficiency of 3.5%. The frequency of transformation for pRK2-based binary Ti vectors was up to 6% for this embryonic axis transformation protocol (Bean *et al.*, 1997). Six of the seven lines had novel sequences with homology to those sequences internal to the T-DNA borders: *uidA* (GUS gene) and *nos-bar* marker gene cassette, based on Southern blot analysis (Figure 4A). Of these seven transgenic lines, three produced spliced transcripts from the intron-containing GUS gene, detected by RT-PCR (Figure 4A); only these three transformed pea lines gave positive GUS histochemical staining. Lines 3 and 4 were self-fertile and produced seed, line 5 produced no seed from the self-crossing but was crossed to a pigmented pea line (JI813). Progeny were analysed for inheritance of GUS activity. In line 3, one of the three seeds had inherited the GUS gene, while for line 4, 11 of the 13 seed were GUS-positive (Table 3), indicating that the T-DNA introduced genes were inherited in a Mendelian manner. Line 4 produced sufficient seed to indicate that there may be multiple independent T-DNA integration sites for this transgenic line.

Figure 4B shows the Southern blot analysis of thirteen transgenic petunias and untransformed parental material. In these experiments modified petunia CHS-A genes were introduced into the petunia genome using pGreen0029 (Table 2). The digest on the transgenic plant DNA displays two fragments corresponding to the endogenous CHS-A gene (at 3 kb and 6 kb), in addition to the fragments from the T-DNA. The 2 kb fragment, in the transgenic lines 4 to 10, is an internal fragment from the T-DNA, and its relative hybridisation intensity to the endogenous fragments is an indication of T-DNA copy number. These transgenic petunia plants are self-sterile, but can be backcrossed to parental material, and the inheritance of the transgene monitored. Four lines that showed phenotypes in the primary transgenic plants were monitored for the inheritance of the T-DNA sequence in their backcrossed progeny. In all cases analysed about half of the progeny contained novel sequence derived from the T-DNA (Table 3). The inheritance of the T-DNA correlated with a post-transcriptional RNA degradation phenotype and, in addition to these plants, three other back-crossed populations showed 50% inheritance of the phenotype.

Figure 4C shows a northern blot of total RNA from ten independent transgenic potato lines derived from transformation experiments with a starch synthase antisense gene (GROT-2) cloned into pGreen0049 (Table 2). Kanamycin-resistant shoots were screened for LUC activity, and 10% (19/183) of these were LUC-positive. An equivalent experiment was performed with a similar 35S-LUC gene construct in pBINLUC (Mullineaux *et al.*, 1990) in which 7.4% (10/135) gave kanamycin-resistant, LUC-positive plants. This direct comparison between the transformation ability of pGreen and the RK2-based binary vectors indicated that transformation efficiency is comparable for both these plasmids for the protocol used for potato tuberdisc transformation. In this pGreen transformation experiment the target gene for antisense was significantly down-regulated in lines 2, 3 and 10, and to a lesser extent in several of the other lines.

Figure 4D shows a Southern blot corresponding to *Dra*I digests of ten independent transgenic tobacco plants generated with pGreen 0049 (Table 2). The digestion of *Dra*I is unaffected by CG or C×G methylation (Nelson and McClelland, 1991), common to the genomic DNA of plants. *Dra*I is absent from the pGreen T-DNA, so fragments identified by homology to the T-DNA sequence represent distinct genetic loci. The filters shown in Figure 4D were hybridised to probes derived from *NptII*, LUC and the pGreen vector backbone. Hybridisation patterns were identical for both the *Npt*II and LUC probes, while the pGreen backbone hybridisation showed no detectable homology to the *Dra*I-digested DNA. The segregation of transgenic tobacco plants transformed

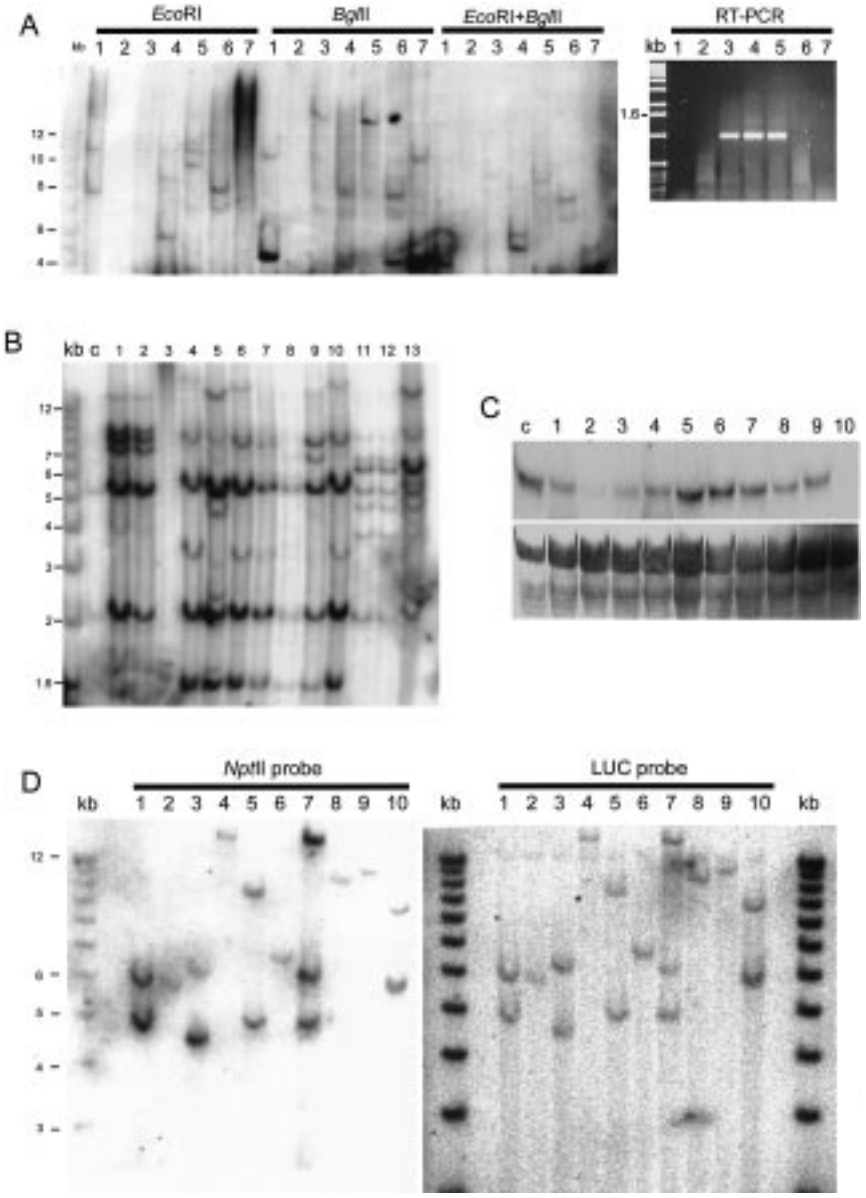

Figure 4. A. Southern blot of genomic DNA from pea plants transformed with pGreen0229 (Table 2) harbouring an intron containing GUS gene. Genomic DNA (5 μg) from seven potential transformants (lanes 1–7) was digested with *Eco*RI, *Bgl*II and *Eco*RI+*Bgl*II, separated on a 0.8% TAE agarose gel at 2 V/cm and hybridised with a T-DNA probe corresponding to GUS and *nos:bar:nos*. RT-PCR on cDNA from the leaves of transformed pea plants (1 to 7). The oligonucleotide primers amplify the GUS gene at either side of the 300 bp intron and give a 1 kb spliced PCR product detected in lanes 3, 4 and 5. B. Southern blot of genomic DNA from petunia plants transformed with a pGreen0029 (Table 2) harbouring petunia *CHS-A* gene constructs. Genomic DNA (5 μg) of from 13 potential transformants (lanes 1–13) and a control untransformed plant (C) was digested with *Hin*dIII and separated as in A. Hybridisation was with a probe derived from the subclone of a 4 kb *Eco*RI fragment of the endogenous gene. C. Northern blot of total RNA from 10 transgenic potato plants harbouring a starch synthase antisense gene in pGreen0049 (Table 2). The top panel shows the suppressed levels of starch synthase mRNA in 3 lines (lanes 2, 3 and 10). The bottom panel shows total RNA loading as determined by staining with 0.03% w/v methylene blue in 0.3 M acetic acid. D. Southern blot of genomic DNA (10 μg) from tobacco plants transformed with pGreen0049 (Table 2). Genomic DNA from each of ten potential transformants (lanes 1–10) was digested with *Dra*I and separated as in A. This filter was probed with *nos-NptII*, LUC and pGreen backbone sequence. No signal was detected for the hybridisation with the non-T-DNA probe.

with pGreen0029 (Table 2) was measured by the inheritance of the kanamycin resistance gene. Seed of self-pollinated regenerant plants were germinated on media containing 50 μg/ml kanamycin. For seven (7/12) independent transgenic plants analysed, the inheritance of kanamycin resistance in 75% of the progeny suggested the integration of the T-DNA into a single genetic locus, four (4/12) lines showed segregation ratios greater than 3:1 and would be candidates for multiple T-DNA loci, and one line had a ratio of ca. 2:1 and insufficient data to ascribe a putative genetic composition.

pGreen Internet site

More detailed information concerning all the methods, manipulations and sequence information used to construct pGreen, pSoup, selectable marker gene and reporter gene cassettes, a catalogue of prebuilt vectors and information on procedures for requesting this plasmid is available on a Web site: http://www.pGreen.ac.uk

Discussion

As outlined in the Introduction, our intention was to develop a small, high-copy and versatile binary vector. By separation of the *ori* and *repA* gene of the pSa locus for replication in *Agrobacterium*, we have been able to produce a basic binary vector of less than 3.5 kb (Figure 1). This includes the pUC replication origin, which permits replication to high copy number in *E. coli*. Using the pBluescript II multiple cloning site (Fritsch *et al.*, 1995), and adding unique restriction sites to the synthetic LB and RB sequences, we have produced a T-DNA which can easily be configured to suit most experimental situations. The availability of a set of commonly used selectable reporter genes, inserted into both *nos* and CaMV 35S expression cassettes, is an integral part of this system's flexibility. These cassettes have been designed for easy insertion into complementary restriction sites internal to the RB and LB and all restriction sites have been removed which would have been duplicated in the pGreen T-DNA cloning sites.

pGreen is unable to replicate in *Agrobacterium* species without the helper plasmid, pSoup, or to be mobilised between bacteria. Along with the intrinsic instability of the pGreen plasmid under non-selective conditions, this enhances the biological safety of this plasmid. In stable transformation procedures pGreen appears to be as efficient as the RK2-based plasmid pBINLUC in producing regenerated shoots containing a T-DNA. Judging from the collected data in this paper, the majority of T-DNA integrations are single loci. The two *Bgl*II sites that flank the T-DNA or the methyl-insensitive *Dra*I are convenient enzymes that address the accuracy of T-DNA integration into the genome (Figure 4). Genomic DNA from transformed plants digested with *Bgl*II should produce fragments larger then the initial T-DNA if the T-strand is nicked at the LB and RB during the T-DNA transfer process; likewise, *Dra*I fragments that contain homology to the T-DNA are generated by restriction sites flanking the T-DNA integration site, and are a good indicator of T-DNA locus number. Data generated on the limited number of transgenic plants assayed and described in this paper indicate there has been no detectable transfer of the pGreen vector backbone into the genome of transgenic plants.

The dual-plasmid system provides additional flexibility centred around the exploitation of pSoup. This plasmid has a multiple cloning site and can act as a platform for further enhancements to *Agrobacterium* technology. Additional *vir* genes have been shown to improve the transfer of larger T-DNAs (Hamilton *et al.*, 1996) and to improve the frequency of gene transfer to cereal species (Hiei *et al.*, 1994; Ishida *et al.*, 1996; Cheng *et al.*, 1997; Tingay *et al.*, 1997). It will be possible to enhance the copy number of key *vir* genes by cloning them into pSoup, thus producing *Agrobacterium* strains with enhanced T-DNA transfer efficiencies. It should also be possible to provide pSoup with a T-DNA, converting it to a T-DNA vector, such that within a single *Agrobacterium* cell the two T-DNA types (one from pGreen and one from pSoup) can co-exist. Co-transformation of both T-DNAs and subsequent segregation will permit the development of marker-free transgenic protocols (Komari *et al.*, 1996).

We have developed an Internet site where extensive information and associated data can be accessed conveniently, and updated accordingly; there is a catalogue containing the cassette combinations already produced, and this list will be updated regularly, so that a particular marker gene combination in pGreen need not be re-built.

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