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Full Length Research Paper

Novel pGreen/pSoup dual-binary vector system in multiple T-DNA co-cultivation as a method of producing marker-free (clean gene) transgenic rice (*Oriza sativa* L) plant

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The possibility of producing marker-free transgenic rice plants using a novel dual binary pGreen/pSoup vectors, in multiple T-DNA co-cultivation, was investigated and demonstrated to be feasible. The T-DNA in pSoup (pRT47) vector was engineered to contain the selection marker hygromycin phosphotransferase (*aphIV*) gene (plus intron in 5′ UTR), and the green fluorescent protein (*gfp*) as a reporter gene both driven by the CaMV35S promoter and the nopaline synthase terminator. T-DNA in the pGreen (pRT18) vector harboured the phosphinothricin acetyl transferase (*bar*), as selection marker gene, and the β- glucuronidase (*gusA*) plus intron as a reporter gene, both driven by the maize 5′ ubiquitin region and the nopaline synthase terminator. Both the pGreen and pSoup plasmids were transformed into *E. coli* strain DH5α using the PEG-transformation technique and into *Agrobacterium* strains AGL1using a freeze-thaw method. AGL1 was then used to transform embryogenic nodular units (ENU), derived from mature seeds of the model rice genotype Nipponbare. Selection on herbicide (PPT) or antibiotic (hygromycin) of co-cultured ENUs led to the production of numerous independently transformed callus clones containing both T-DNAs from the selected and unselected vector. While co-transformation frequencies were 71% and 80% for the hygromycin only and herbicide (PPT) only selection, respectively, data showed that co-expression frequency is most useful for the production of marker free transgenic rice. About half (50%) of the independent transgenic plant lines contained at least one unlinked T-DNA integration. In this work, we showed for the first time, that the novel dual-binary pGreen/pSoup can efficiently produce marker-free transgenic rice.

Key words: pGreen/pSoup, dual-binary vectors, T-DNA co-cultivation, marker-free, clean gene, transgenic, rice, hygromycin, PPT.

INTRODUCTION

Various antibiotic regimens as selection agents are routinely used to identify transformation events. With the exception of the selectable markers that provide herbicide tolerance as gene of interest, the selectable marker genes generally have little agronomic value and could be considered a nuisance after transformation event. They are thus generally unwanted, especially in sequential transformation experiments or in transgenic crops. It is also generally agreed that selection marker genes may pose bio-safety problems hence marker-free transgenic plants are highly desirable.

For more than a decade, there has been consistent and considerable progress in *Agrobaterium* -based

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technologies for the transformation of recalcitrant crop species.

Such advances are due, in part, to the improvement of Ti vector design (see Hellens and Mullineaux, 2000 for review). While important improvements in Ti vectors used for cereal crop transformation have been made (Hei et al.,1994; Komari et al.,1996; Wang et al., 1998; McCormac at al., 1999), there is still a demand for user-friendly vector systems offering small plasmid size, extensive multiple cloning sites, transformation selection flexibility, a flexibility to adapt to specific requirements (such as transfer of large pieces of DNA and transposon-based transgene reposition), allowing multiple T-DNA transfer and leading to high transformation frequencies and possibility of marker-free transgenic plant production. In this study, we tested a new dual-binary vector system pGreen/pSoup (Hellens et al., 2000) for generating marker-free transgenic rice plant.

pGreen is a small (3 kb) Ti binary vector able to replicate in *Escherichia coli* but unable to replicate in *Agrobacterium* without the presence of another binary plasmid, pSoup, in the same strain. pSoup provides replication functions in trans for pGreen and further information on the pGreen/pSoup system can be found on the internet web site, http://www.pgreen.ac.uk.

Several approaches have been developed to remove or eliminate selectable marker genes from transgenic plants (for reviews see Yoder and Goldsbrough, 1994; Ebinuma et al., 2001; Hare and Chua, 2002) and such approaches include (1) co-integration of the gene of interest and the selectable marker gene at unlinked loci (de Framond et al., 1986), (2) excision of the selectable marker gene by site-specific recombination (Dale and Ow, 1990), (3) transposon-mediated repositioning of the gene of interest (Goldsbrough et al., 1993), and (d) excision of the selectable marker gene by homologous recombination (Zubko et al., 2000). Approach (1) above was the first to be implemented in plants (de Framond et al., 1986) and has been used in a wide range of species (Mc Knight et al., 1987; De Neve et al., 1997) including crops (De Block and Debrouwer 1991; Komari et al., 1996; Xing et al., 2000). Generally, it relies on *Agrobacterium tumefaciens*-mediated co-transformation of different transgenes carried by different T-DNAs. The different T-DNAs can either been present in one-vector/one-strain (Komari et al., 1996; Xing et al., 2000), two-vectors/one-strain (de Framond et al., 1986; Daley et al., 1998) or two vectors/two strains (McKnight et al., 1987; De Block and Debrouwer, 1991; De Neve et al., 1997). This multiple T-DNA approach differs from the other three in that unlinked integration of the different transgenes is achieved directly during the plant cell transformation process and does not require further transgenic locus alteration. The simplicity of this system is a great advantage but it also bears some limitations. The characteristics of the original integration sites may include unwanted DNA sequences (T-DNA and vector sequences including borders) or rearrangements (plant genomic DNA repeats or truncated T-DNA) which are retained (Cotsaftis et al., 2002). The efficiency of this strategy also relies on the understanding of the mechanisms underlying T-DNA integration and T-DNA linkage in plants. Although the integration of T-DNAs in all possible configurations has been well documented (Jorgensen et al., 1987; Koncz et al., 1994; De Neve et al., 1997), to date there is only partial information available on transgenic locus composition and T-DNA linkage configuration in populations of transformed plants. Most previous studies on T-DNA linkage, with few exceptions (Bhattacharyya et al., 1994; Cluster et al., 1996; Lu et al., 2001; Matthew et al., 2001), were based on the molecular analysis of primary transformants (Southern blot analysis of T_0 plants) combined with the segregation analysis of the transgene phenotype. Previously, we showed that such an approach is prone to underestimate transgenic locus number and therefore to present a distorted view of T-DNA linkage groups in plants (Vain et al., 2003). Recently, it has been shown that 30–60% of the plants transformed with different T-DNAs can contain several types of T-DNA inserts (Lu et al., 2001; Miller et al., 2002) with some inserts containing either linked or unlinked T-DNAs or a mixture of both. The further development of the multiple T-DNA strategy relies, therefore, on a better understanding of locus constitution and T-DNA linkage in populations of transgenic plants. Recent studies in a large population of multiple T-DNA transformed rice plants have also produced new insights on locus composition and T-DNA linkage configurations (Afolabi et al., 2004).

In cereals, the development of marker-free transgenic plants has been hampered because efficient *Agrobacterium*-based transformation technologies have only recently been developed (Hiei et al., 1994). The multiple T-DNA approach was also the first to be implemented in cereals (Komari et al., 1996) and is now used to produce marker-free transgenic rice (Komari et al., 1996; Lu et al., 2001), barley (Matthew et al., 2001) and maize (Miller et al., 2002) plants. Rice plants containing only a rice ragged stunt virus resistance transgene have been produced using the one-vector/one-strain version of this strategy (Lu et al., 2001). The multiple T-DNA approach is likely to be one of the preferred type of “clean-gene” technology to produce transgenic cereal crops free of selectable marker genes in the near future.

The use of the novel pGreen/pSoup dual binary vector system in co-transformation of multiple T-DNAs to produce marker-free transgenic rice plants requires that the selection marker gene be carried on one T-DNA, and the gene of interest on the other and both however must be co-transformed, co-integrated into the plant genome and co- expressed. It was envisaged that during meiosis, the two T-DNAs would, if they were not completely linked genetically, be separated by

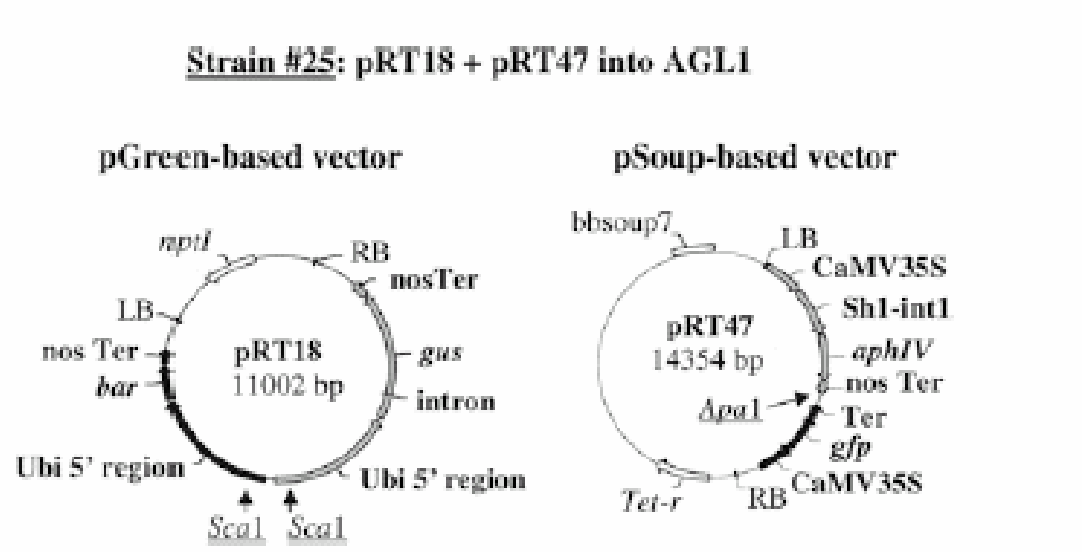


Figure1. pGreen/pSoup-based vectors used for Rice transformation.Gene abbreviations are detailed in the experimental procedures. *Agrobacterium* Srain No. 25 contains pRT18 and pRT47 binary vectors. The T-DNA of PRT18 (pGreen-based) contains the bar and gus genes. The T-DNA of pRT47(PSoup-based) contains the aphIV and gfp genes. Probes rfor backbones sequences (nptI and BBsoup7) approximately 1kb from the LB of each T-DNA are represented. In *Agrobacterium tumefaciens* psoup-based vectors provide replication in trans for pGreen-based vectors(Hellens et al. 2000).

segregation and some haploid daughter cells would contain only the genes from either (and not both) of the T-DNAs. The probability of nonlinkage and segregation of the two T-DNAs could be greatly enhanced if both the frequency of co-transformation of the T-DNAs is high and their relative position in the plant genome is sufficiently far apart to allow separation.

Since T-DNA replication always precede transfer and integration into the plant genome, pGreen dependent replication on pSoup, which is the unique feature of these novel vectors, assures that replication of both vectors is not independent and was exploited in this study to produce marker free transgenic rice plants.

It was necessary, therefore, to independently select for each plasmid alone and, experimentally determine how often the unselected plasmid was co-transformed with the selected one. It was envisaged that the plasmid that had the higher co-transformation efficiency when the other plasmid was selected, would be the ideal one to carry the gene of interest, because the chances and possibility of producing marker-free transgenic plants would be enhanced.

In the past, the pGreen/pSoup system has mostly been used for the transformation of dicotyledonous species using a single T-DNA present on the pGreen vector only (Hellens et al., 2000). In this study, we engineered both pGreen and pSoup vectors to contain each a different T-DNA. Consequently, two parallel rice transformation experiments, in which each selection marker gene on different T-DNAs was selected for were conducted. We then assessed the segregation of the co-transformed and co-expressed T-DNAs in T_1 rice plants.

MATERIALS AND METHODS

PGreen/pSoup-based vectors

pGreen- based and pSoup- based dual binary vectors and *Agrobacterium* strain #25 used for rice transformation (Figure 1) were constructed as previously reported (Vain et. al., 2003, Afolabi et al., 2004). pRT18 (pGreen-based) contained the phosphinothricin acetyl transferase (*bar*) gene, a selection marker gene, and the β- glucuronidase gene (*gusA*) plus intron as a reporter gene, both driven by the maize 5′ ubiquitin region and the nopaline synthase terminator (Vain et al., 2003). pRT47 (pSoup-based) harbored the hygromycin phosphotransferase (*aphIV*), (plus intron in 5′ UTR) gene as the selection marker gene, and the green fluorescent protein (*gfp*) gene as a reporter gene both driven by the CaMV35S promoter and the nopaline synthase terminator (Vain et al., 2003). As no additional virulence genes are present in the vectors' backbone, neither pRT18 nor pRT47 are super binary vectors (Hiei et al., 1994).

Plasmids were transformed into *E. coli* strain DH5α using the PEG-transformation technique and into *Agrobacterium* strain AGL1 using a freeze-thaw technique. As detailed in Figure 1, *Agrobacterium tumefaciens* strain no. 25 contains pRT18 (pGreen-based) and pRT47 (pSoup-based).

Rice transformation procedures

Loose embryogenic calli derived from mature seeds of rice (*Oryza sativa* L.) variety Nipponbare were used for transformation as previously described (Vain et al., 2003; Afolabi et al., 2004) Briefly, the embryos were aseptically removed from sterilized seeds and

plated onto NBm medium for 3 weeks in the dark at 25°C. Loose embryogenic translucent globules (U), around 1 mm in size, were separated by rolling the callus grown from the original embryo onto the gelling agent. Globules were cultured for an additional 10 days on fresh NBm medium (~100 globules per plate) to produce embryogenic nodular units (ENUs; Bec et al., 1998), used as targets for transformation. Culture plates containing ENUs were flooded with bacterial suspension at an OD=1 (600 nm) for 5 min. ENUs were blotted and co-cultivated on NBm medium supplemented by 200 µM acetosyringone for 2 days. After co-culture, ENUs were put onto selection medium (NBm medium containing 5 mg/l phosphinotricin (PPT) and/or 50 mg/l hygromycin for 2 weeks, then subcultured for an additional 3 weeks. Timentin (150 mg/l) was added to all selection media and L-glutamine was removed from culture media with PPT. Plants were regenerated by successive transfer onto pre-regeneration (PRm), regeneration (RNm) and germination selection media (Vain et al., 2003). Only one plant was regenerated from each original ENU (explant) to guarantee that each plant represented an independent transformation event. Transformed plants were transferred to a controlled environment room for growth to maturity. All transgenic plants produced were analysed to ensure the study of series of random, independent transformation events, with the widest spectrum of expression for the non-selected *gusA* and *gfp* genes. Transgenic plants were cultured, sampled and analysed using a standard operating procedure (SOP) (James et al., 2004).

Analysis of GUS and GFP activity

Fluorometric analysis for β-glucuronidase activity was carried out on leaf tissue from rice plants at the five-leaf stage, following the protocol of Jefferson (1987). Fluorescence was measured using a Titertek Fluoroskan II after 0, 30 and 60 min incubation. Each assay was performed in triplicate. Protein content was determined using a Bio-Rad protein assay kit. Data were expressed as pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of extracted protein. The background activity (33±4 pmol MU min^{-1} mg^{-1} protein) was subtracted from all fluorometric *GUSA* measurements as described by Vain et al., 2002.

Visual detection of GFP fluorescence was performed using a MZ6 Leica dissecting microscope with a fluorescent module (Leica no. 10 446093) and appropriate wavelength filters (425/60 nm excitation filter, 470 nm dichromatic beam splitter and a G6457 emission barrier filter) over a high voltage mercury lamp.

Detection of transgenic plants by the polymerase chain reaction

DNA was isolated and polymerase chain reaction (PCR) reactions were carried out as previously described (Vain et al. 2002). Five primers sets were used on the DNA samples: (1) one to amplify the 1,200 bp single copy rice RFLP probe C213 (forward: 5′-AAAGGACCGGAATGACCACAA-3′; reverse: 5′GAATGAACCACG CCCAAGAGT-3′) in order to ensure that each DNA sample was suitable for PCR amplification, (2) another to amplify a 421 bp fragment of the *bar* gene (forward: 5′-GGTCTGCACCATCGTCAAC C-3′; reverse: 5′-GTCATGCCAGTTCCCGTGCT-3′), (3) another to amplify a 1,013 bp UBI::*gus* fragment (forward: 5′-GGGCGGTCGT TCATTC-3′; reverse: 5′-TTCGGCGTGGTGTAGAGC-3′), (4) another to amplify a 727 bp fragment of the *aphIV* gene (forward: 5′-ACTCACCGCGACGTCTGTCG-3′; reverse: 5′-GCGCGTCTGCT GCTCCATA-3′) and (5) another to amplify a 527 bp fragment of the *gfp* gene (forward: 5′-GGAGAGGGTGAAGGTGATGCAA-3′; reverse: 5′-GGGCAGATTGTGTGGACAGGTA-3′).

Southern analysis

Genomic DNA extraction and Southern analyses were performed on primary transformed rice plants (T_0) as previously described (Vain et al., 2003). Membranes were hybridized with the following probes: 549 bp of the *bar* gene, 701 bp of the *gusA* gene, 981 bp of the *aphIV* gene, 737 bp of the *gfp* gene, 421 bp of the pGreen backbone (888 bp from the left border) or 464 bp of the pSoup backbone (1,216 bp from the left border). The filters were analysed by using a Typhoon 8600 phospho-imager and the Typhoon Scanner Control version 1.0/ImageQuant version 5.1 software (Molecular Dynamics).

Dot blot analysis

The Qiagen DNeasy 96 high-throughput DNA isolation kit was used for genomic DNA extraction from rice plants. The dot blotting procedure was performed using 190 µl of the extracted DNA (approximately 1 µg) and the BIORAD Bio-Dot Micro-filtration apparatus following the manufacturer's protocols and recommendations. Membranes were hybridised sequentially with probes for the *gusA, bar*, *aphIV* and *gfp* genes, for the pGreen and pSoup backbones (as described above) and for the R2272 rice RFLP genomic probe.

Transgene inheritance and segregation of transgene phenotypes

T_1 seeds were obtained by self-pollination of primary transformed rice (T_0) plants. Segregation analyses were conducted by germinating seeds on MSR6 medium (Vain et al., 2002) without selection. Transgene expression in the T_1 embryos or seedlings was assessed qualitatively by histochemical *GUSA* staining (Jefferson et al., 1987), or by observation of GFP fluorescence in at least 64 random T_1 seedlings from each independently transformed T_0 plant. All T_1 plant progeny not expressing either the *gusA* or gfp transgenes were analysed by PCR or dot blot for the presence of the *bar*, *gusA*, *aphIV* and *gfp* genes. All DNA samples were initially tested for the rice probe R2272 to confirm that the extracted DNA samples were suitable for dot blot analysis.

Statistical analyses

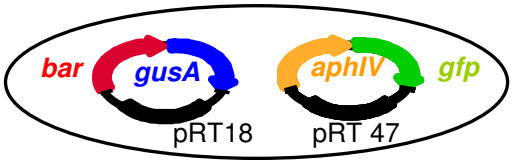
Statistical analyses, following the requirements of each test, were performed using Minitab 13.1 or Genstat 5 software. Data sets were compared using ANOVA or the paired t-test. Observed segregation ratios were compared to Mendelian models using χ2 analysis.

RESULTS AND DISCUSSION

Generation of a population of transgenic rice plants

Rice plants were transformed using the *Agrobacterium* strain no. 25 containing the pGreen-based pRT18 vector (T-DNA containing the *bar* and *gusA* genes) and the pSoup-based pRT47 vector (T-DNA containing the *aphIV* and *gfp* genes) (Figure 1). Three selection regimes were applied during the transformation process with *Agrobacterium* strain no. 25: (a) dual selection of pGreen and pSoup T-DNAs using phosphinothricin (PPT) and

Table 1. Summary of results of transformation experiments using different T-DNA selection regimes.



Selection regimen	pRT18 (PPT)	pRT47 (HYGRO)
Transformation efficiency	1%	21%
No of independent plant lines produced	10	185
No of co-transformed plant lines	80% (8/10)	71% (131/185)
No of plant lines co-expressing transgenes	88% (7/8)	24% (32/131)
No of plant lines studied in T_1	3	9
Unlinked lines	100% (3/3)	44 % (4 /9)
Unlinked loci	63% (5/8)	33% (7/21)
Frequency of loci appearance		
"G-S" locus (linked pGreen and pSoup T-DNA)	38% (3/8)	67% (14/21)
"G locus" (pGreen T-DNA alone)	50% (4/8)	14% (3/21)
"S locus" (pSoup T-DNA alone)	12% (1/8)	19% (4/21)

aphIV, hygromycin phosphotransferase gene; ***bar***, phosphinothricin acetyl transferase gene; ***gusA,*** β-glucuronidase gene ***gfp***, green fluorescent protein gene.

hygromycin, The dual TDNA selection regime was used to produce a large and random population of plants co-transformed and co-expressing all the transgenes present in the pGreen and the pSoup T-DNAs. This strategy was designed to by-pass the limitations of post-transformation screening for co-transformed and co-expressing lines in experiments designed to produce marker-free transgenic plants. The dual selection was designed to provide insights into locus composition and T-DNA linkage configurations in population of rice plants transformed with multiple T-DNA. The results of this are detailed elsewhere (Afolabi et al., 2004). (b) Selection of pSoup T-DNA only, using hygromycin alone. (c) Selection of pGreen T-DNA only, using PPT alone.

The single T-DNA selection regimes were used to directly mimic experiments designed to produce marker-free transgenic plants. 189 independently transformed transgenic rice plant lines were produced (Table 1). Transgene copy numbers and the presence of backbone sequences were determined in 12 random T_0 plants, each representing an independently transformed plant line (nine lines with pSoup T-DNA selection only, three lines with pGreen T-DNA selection only). Although transgenic locus composition and T-DNA linkage configuration were determined in these 12 lines by assessing the presence and expression of the four transgenes in 768 T_1 progeny plants, and detailed elsewhere (Afolabi et. al., 2004), the aim of this particular work was to show that pGreen/pSoup dual binary vectors can efficiently produce marker-free transgenic rice plants.

Single T-DNA selection: locus constitution and TDNA linkage configuration

Single T-DNA selection was undertaken using PPT only (selecting for the *bar* gene present on pGreen-based pRT18) or using hygromycin only (selecting for the *aphIV* gene present in pSoup-based pRT47). Only a limited number of lines were produced by PPT selection, and the results could not be interpreted statistically. However, a large-scale experiment was undertaken using pSoup TDNA selection alone and the transgene presence and expression were analysed in T_0 plants as previously described (Afolabi et al., 2004). Around 70% (131/185) of the lines were co-transformed with the pGreen and pSoup T-DNAs but only 24% (32/131) of the co-transformed lines co-expressed all four transgenes (Figures 2a,b; Table 1). The remaining co-transformed

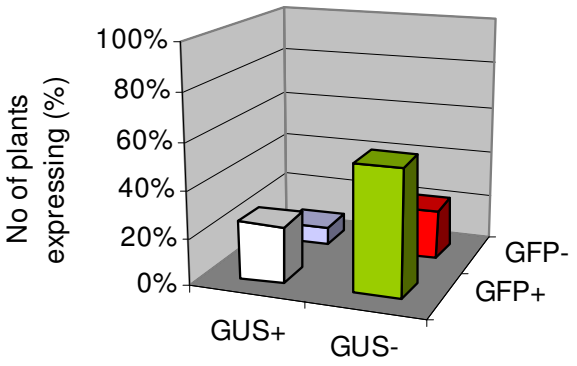


Figure 2a. Co-expression of pGreen and pSoup transgenes when only the pSoup T-DNA was selected.

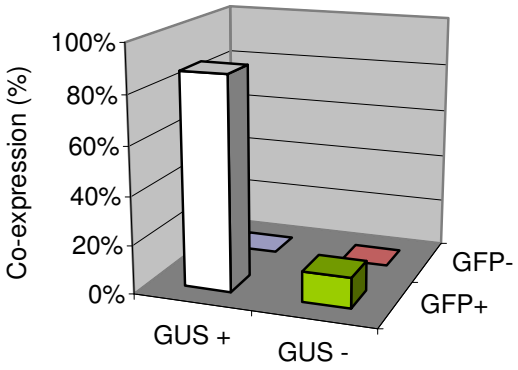


Figure 2b. Co-expression of pGreen and pSoup transgenes when only pGreen plasmid was selected.

lines were either non-expressing for *gfp* (11%), or *gus* (35%) or both genes (30%). Overall, about 29% (53/185) of the lines did not express the *gfp* gene (present in the selected pRT47 T-DNA).

However, 65% (85/131) of the lines did not express the *gusA* gene which is significantly higher than when pRT18 T-DNA was selected for (12/208=6 during dual selection, χ2: P<0.05) (Afolabi et al., 2004). Thus in around two-thirds of the co-transformed lines, the transgenes present in the unselected T-DNA were non-expressing.

This could be due to either non-intact expression units, transcriptional interference from adjacent endogenous plant promoters or silencing at the transcriptional or post transcriptional levels (Bhattacharyya et al., 1994; Dong et al., 2001) of the transgenes present in the unselected T-DNA. Comparable levels of co-transformation (70–90%; Lu et al., 2001; Matthew et al., 2001) and co-expression (30–50%; De Block and Debrouwer, 1991; Komari et al., 1996; Daley et al., 1998) were previously observed for transgenes carried by different binary vectors. This shows that in plants transformed with different TDNAs, co-transformation is less of a limiting factor for the development of "clean-gene" technology than the constraint of co-expression of the non-selected gene(s). In total, nine lines obtained using pSoup T-DNA selection only, and three lines obtained using pGreen T-DNA selection only, co-transformed and co-expressing all transgenes present in pRT18 and pRT47.

T-DNAs, were randomly chosen. Using earlier protocol (Afolabi et al., 2004), transgene molecular and expression analyses of T_0 and T_1 plants for each of the 12 lines studied were conducted (i.e. 727 T1 plants studied representing 1,454 and 1,005 phenotyping and genotyping analyses, respectively). Around 58% (7/12) of the lines contained a "G locus" or an "S locus" while overall 41% (12/29) of the loci contained single-type T-DNA(s). Progeny plants (T_1) containing a single type of T-DNA could therefore be obtained by selecting either for pGreen-based pRT18 TDNA or for pSoup-based pRT47 T-DNA. This demonstrates that the pGreen/pSoup dual binary vector system is well suited for the production of marker-free transgenic plants and for the development of efficient "clean-gene" technology in rice.

Segregation of the two T-DNAs and marker-free transgenic rice plants

In both the pRT47 and pRT18 selection, segregation analysis data showed that there were some progeny plants that contained only one T-DNA after segregation; thus providing evidence that the novel pGreen/pSoup dual binary vectors can, indeed, produce marker-free ("clean gene") transgenic rice plants. For the pSoup (pRT47) selection, Figures 3a and 3b show examples of some progeny plants that had transgene(s) from one T-DNA only (Figure 3a) nonlinkage) and, other (Figure 3b), with both T-DNAs (linked) respectively. In Figure 3a, the dot blot of samples in D12–F3 showed non-linkage and, the separation of the pGreen T-DNA and the pSoup T-DNA was as follows: pGreen T-DNA only were samples D9, 10 and E12 and, pSoup T-DNA only were samples D4, 5, 7, 8, 11 E5. Sample E7 contained truncated pSoup T-DNA as evidenced by the presence of gfp gene but no aphIV gene both of which were present on the same pSoup T-DNA. Figure 3b showed a one-locus linkage of both the pGreen and pSoup T-DNAs as evidenced by the samples in D1 – D5. All samples contained or lack both T-DNAs.

For the pGreen (RT18) selection, Figures 4a and 4b also showed examples of some progeny plants that had transgene(s) from one T-DNA only (Figure 4a) nonlinkage and, other (Figure 4b), with both T-DNAs (linked. The segregation/separation of T-DNAs (transgenes) are as follows:

pGreen only: B9, B11, C1, C2, C3, C4, C5, D2, D3, D6, D9, D10
pSoup only: B6, B12, C7, C8, C10, C11, D4, D5, D7, D8, D11

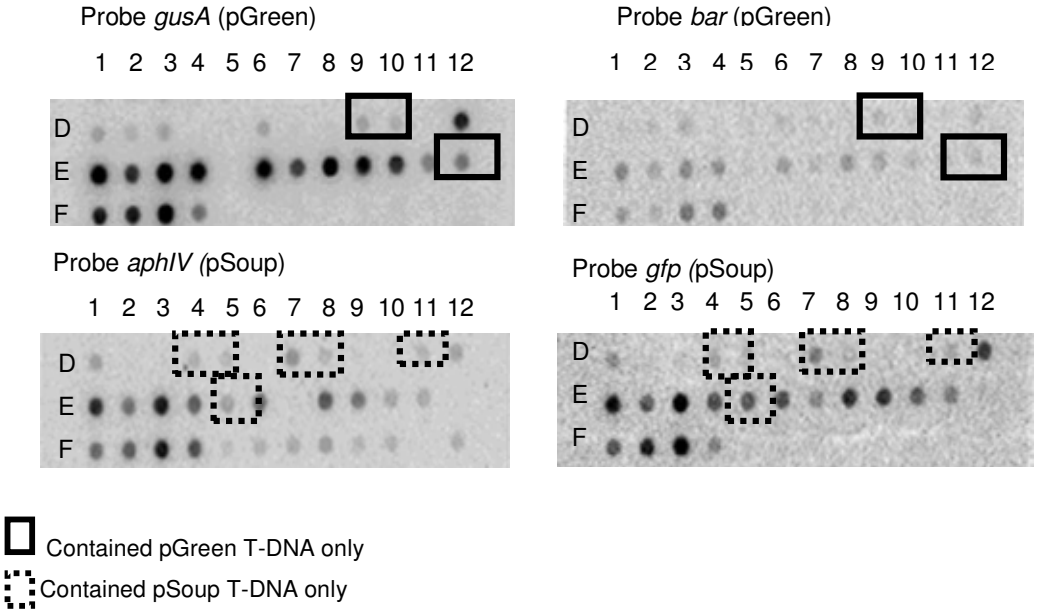


Figure 3a. Example of progeny plants containing transgene(s) from one T-DNA only after the segregation of the multiple T-DNAs co-transformed T_0 plants. G+; genotype contained transgene(s) from pGreen plasmid G-; genotype did not contain transgene(s) from pGreen plasmid. S+; genotype contained transgene(s) from pSoup plasmid; S-; genotype did not contain transgene(s) from pSoup plasmid. Probe R2272 is a RFLP rice probe; positive indicated the suitability of the DNA used for dot blot analysis.

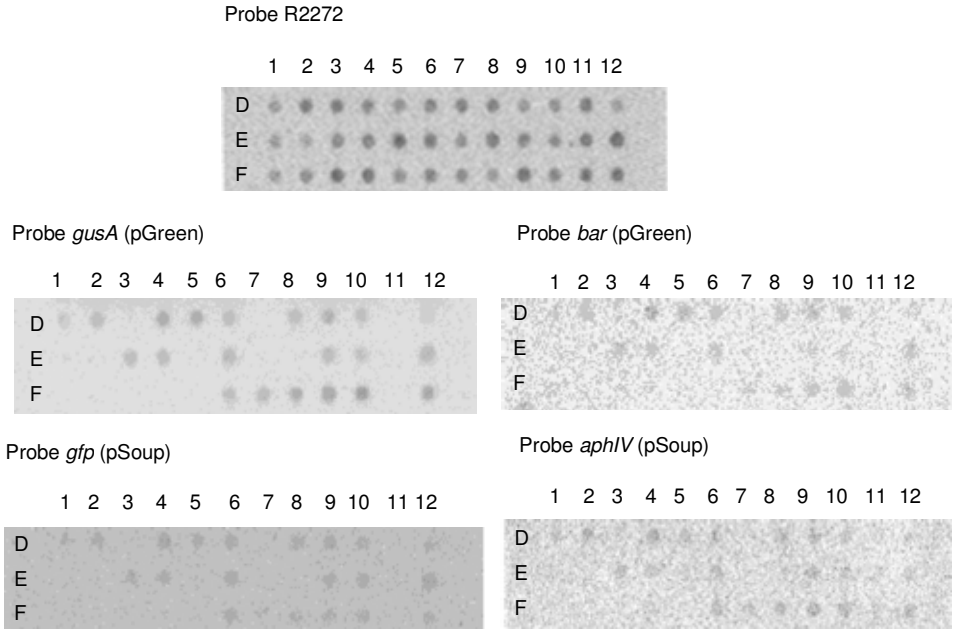


Figure 3b. Example of progeny plants containing transgene(s) from both T-DNAs (linked) after the segregation of the multiple T-DNAs co-transformed T0 plants. G+; genotype contained transgene(s) from pGreen plasmid G-; genotype did not contain transgene(s) from pGreen plasmid. S+; genotype contained transgene(s) from pSoup plasmid; S-; genotype did not contain transgene(s) from pSoup plasmid. Probe R2272 is a RFLP rice probe; positive indicated the suitability of the DNA used for dot blot analysis. G+; genotype contained transgene(s) from pGreen plasmid G-; genotype did not contain transgene(s) from pGreen plasmid. S+; genotype contained transgene(s) from pSoup plasmid; S-; genotype did not contain transgene(s) from pSoup plasmid. Probe R2272 is a RFLP rice probe; positive indicated the suitability of the DNA used for dot blot analysis.

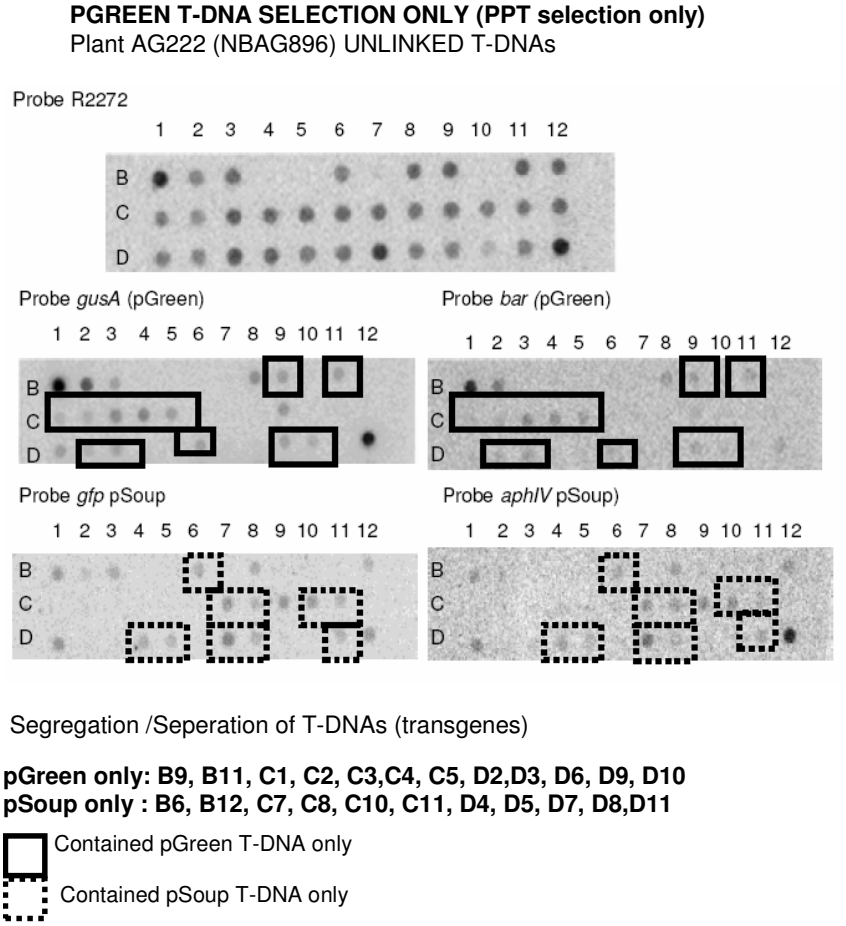


Figure 4a. Example of progeny plants containing transgene(s) from one T-DNA only after the segregation of the multiple T-DNA co-transformed T_0 plants. G+; genotype contained transgene(s) from pGreen plasmid G-; genotype did not contain transgene(s) from pGreen vector. S+; genotype contained transgene(s) from pSoup vector; S-; genotype did not contain transgene(s) from pSoup vector. Probe R2272 is a RFLP rice probe; positive indicated the suitability of the DNA used for dot blot analysis.

Finally, Figure 4b showed an example of linked T-DNAs when pGreen was selected.

The overall efficiency for the production of a transgenic locus containing a single type of “active” T-DNA is estimated to be around 9% (co-transformation 139/195= 71% × co-expression 39/139=28% × 55/127=43% unlinked loci; Table 1). The percentage of lines containing at least one “active” single T-DNA type locus is estimated to be about 10% (co-transformation 139/195=71% × co-expression 39/139=28% × 31/62=50% unlinked lines; Table 1). Backbone transfer (in 53%–66% of the loci for pRT18 and pRT47) should further reduce these overall efficiencies to about 5%. It is somewhat difficult to compare the results obtained in this study with previously published data as the assessment of “clean-gene” technology performances is directly linked to the accurate assessment of transgenic locus number and TDNA linkage configurations. In the past, genotyping of large populations of plants transformed with different TDNAs and their progeny have rarely been conducted (Matthew et al. 2001; Lu et al., 2001). However, unlinked T-DNA integration in 16% (15/95) and 50% (18/39) of independently transformed plant lines has been reported in barley (Matthew et al., 2001) and rice (Lu et al., 2001) respectively, using the one-vector/one-strain approach. It is therefore possible that the two-vectors/one-strain approach that we used could limit “clean-gene” efficiency when compared to the one-vector/one-strain approach. Strategies to counter-select backbone transfer would constitute one of the first optimization steps of “clean-gene” technologies based on plant transformation with multiple T- DNAs. Alternatively, new binary vectors could be designed. Each binary vector could be regarded as not one but two “transferable” DNA fragments separated by borders. One fragment (probably the T-DNA itself), could harbor only the gene(s) of interest (without any selectable marker gene), while the backbone could contain both of the plant and bacterial selectable marker

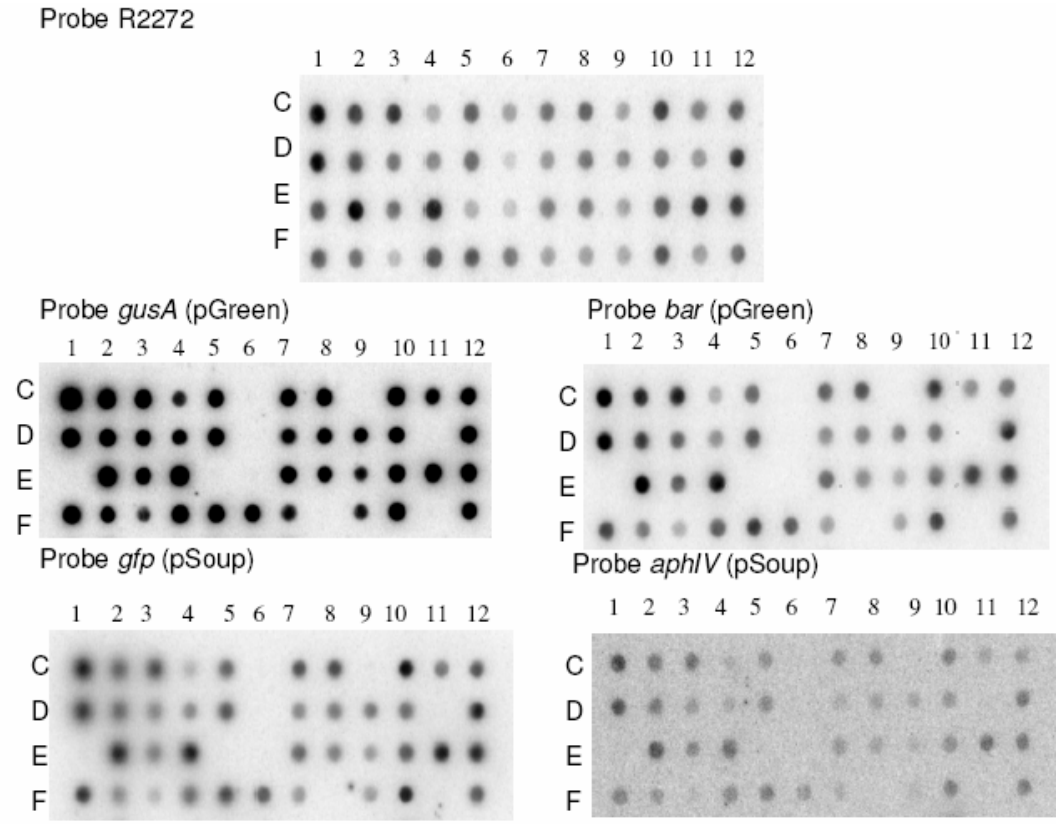


Figure 4b. Example of progeny plants containing transgene(s) from both T-DNAs (linked) after the segregation of the multiple T-DNA co-transformed T_0 plants. G+; genotype contained transgene(s) from pGreen plasmid G-; genotype did not contain transgene(s) from pGreen vector. S+; genotype contained transgene(s) from pSoup vector; S-; genotype did not contain transgene(s) from pSoup vector. Probe R2272 is a RFLP rice probe; positive indicated the suitability of the DNA used for dot blot analysis.

genes as well as additional genes (*vir* etc.). The recovery of transgenic plants would be assured by co-transformation of the TDNA and backbone sequences in a linked or unlinked fashion. Progeny plants free of selectable marker genes would be obtained by segregation of loci containing only the T-DNA. In this situation the dual binary vector system pGreen/pSoup could become a four component super binary vector system.

In conclusion, clean gene (marker-free) technology, using a novel dual binary pGreen/pSoup vectors in multiple T-DNA co-cultivation, was investigated and demonstrated to be feasible. This can be achieved either with the pGreen or pSoup vector carrying the target (interest) gene. The pGreen/pSoup dual-binary vector events were clearly not independent. This dual-binary-vector system is unique in that pGreen depends on pSoup to replicate, thus the possibility of co-transformation is increased. However, as seen from the data obtained here, the limiting factor in producing marker-free transgenic rice is co-expression, although co-transformation and co-integration of the selection marker-gene and the gene of interest into the plant genome must first occur.

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