

Generation of Marker- and Backbone-Free Transgenic Potatoes by Site-Specific Recombination and a Bi-Functional Marker Gene in a Non-Regular One-Border *Agrobacterium* Transformation Vector

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Abstract A binary vector, designated PROGMO, was constructed to assess the potential of the *Zygosaccharomyces rouxii* R/Rs recombination system for generating marker- and backbone-free transgenic potato (*Solanum tuberosum*) plants with high transgene expression and low copy number insertion. The PROGMO vector utilises a constitutively expressed plant-adapted R recombinase and a *codA-nptII* bi-functional, positive/negative selectable marker gene. It carries only the right border (RB) of T-DNA and consequently the whole plasmid will be inserted as one long T-DNA into the plant genome. The recognition sites (*Rs*) are located at such positions that recombinase enzyme activity will recombine and delete both the bi-functional marker genes as well as the backbone of the binary vector, leaving only the gene of interest flanked by a copy of *Rs* and RB. Efficiency of PROGMO transformation was tested by introduction of the *GUS* reporter gene into potato. It was shown that after 21 days of positive selection and using 300 mg l⁻¹

5-fluorocytosine for negative selection, 29% of regenerated shoots carried only the *GUS* gene flanked by a copy of *Rs* and RB. The PROGMO vector approach is simple and might be widely applicable for the production of marker- and backbone-free transgenic plants of many crop species.

Keywords Backbone-free transgenic plants · *codA-nptII* · Marker-free transgenic plants · Recombination · R/Rs · Solanaceae

Introduction

Generation of marker- and backbone-free transgenic plants responds to public concerns on the safety of genetically modified (GM) crops and supports multiple transformation cycles for transgene pyramiding.

Transformation of plant cells occurs at a very low frequency. For identifying those cells that have integrated the DNA into their genome, approximately 50 different selection systems have been developed over the past several years. Despite of the large number of systems, marker genes that confer resistance to the antibiotics kanamycin (*nptII*) and hygromycin (*hpt*), or the herbicide phosphinothricin (*bar*) were used for most plant research and crop development. There are basically two strategies to eliminate

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the markers. The simplest is the co-transformation of genes of interest with selectable marker genes followed by the segregation of the separate genes through sexual crosses. Co-transformation has been accomplished in a number of ways including co-inoculation of plant cells with two *Agrobacterium* strains each containing a simple binary vector, dual binary vector systems, and modified two-border *Agrobacterium* transformation vectors (Breitler et al., 2004; Huang et al., 2004; Miki and McHugh, 2004 for a review). The other strategy is the use of site-specific recombinases, under the control of inducible promoters, to excise the marker genes. Successful use of the Cre/lox, FLP/FRT or R/Rs systems has been reported in different plant species, in which Cre, FLP and R are the recombinases, and lox, FRT, and Rs are the recombination sites, respectively (Hare and Chua, 2002 for a review).

Potato is a cross-pollinating, highly heterozygous, vegetatively propagated crop. Thus elimination of marker genes via sexual crosses would be difficult in this species. Strawberries are also propagated vegetatively. Recently, effective production of marker-free transgenic strawberry plants was reported using a plant-adapted inducible R recombinase gene and a bi-functional, positive/negative selectable marker to reduce the appearance of chimeras due to incomplete DNA excision (Schaart et al., 2004). The positive selection was provided by *nptII* whereas the negative selectable marker was the *codA*, a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU). *CodA* was employed first by Gleave et al. (1999) for the production of marker-free transgenic tobacco plants, albeit at low frequency. No attempts have been published on the use of a recombinase method for marker elimination in potato.

De Vetten et al. (2003) reported transformation of potato without the use of selectable markers. The best results were obtained with the potato variety Karnico using the *Agrobacterium tumefaciens* strain AGL0 that exhibits extremely high transformation efficiency because it contains a DNA region originating from a super

virulent *A. tumefaciens* strain. In this experiment, approximately 5,000 regenerated shoots were isolated and analysed by PCR. Transgenic lines were obtained with an average frequency of 4.5%. However, vector backbone sequences were transferred along with the gene of interest in 60 out of the 99 transgenic lines and only 10 vector-free lines contained a single T-DNA insertion.

The isopentenyl transferase (*ipt*) gene that leads to cytokinin overproduction and results in transgenic shoots with abnormal shoot morphology can also be used as a selectable marker (Sugita et al., 2000; Endo et al., 2002). In this case, appearance of normal-looking plants emerging from abnormal tissues indicates excision of the *ipt* gene resulting in marker-free plants. *Ipt* selection was combined with a plant-derived T-DNA-like P-DNA fragment and used to generate marker- and backbone-free potato lines in a dual binary vector system with negative selection provided by *codA* against *nptII* marker gene integration. Using this highly efficient way of selection hundreds of marker- and backbone-free Ranger Russet potato plants displaying reduced expression of a tuber-specific polyphenol oxidase gene were produced by Rommens et al. (2004). However, the copy number of the insertions was not investigated in this experiment.

Here we describe the construction and application of an improved marker- and backbone-free transformation system based on a plant-adapted R recombinase and a *codA-nptII* bi-functional selectable marker gene. Due to the presence of a single T-DNA border the whole streamlined binary vector is integrated into the plant genome. The location and orientation of the *Rs* sites allows for a subsequent deletion by recombinase activity of the whole vector DNA, except for the gene of interest, from the host genome. We show that constitutive expression of the recombinase with temporary exposure of the *Agrobacterium* infected leaves to kanamycin followed by a negative selection against the presence of the marker gene allows for a direct selection of low copy marker- and backbone-free insertions with a high level of expression of the gene of interest in potato.

Materials and methods

Construction of Binary Plasmids

The synthetic version of the *R* recombinase gene of *Zygosaccharomyces rouxii* (Schaart et al., 2004) containing the *ST-LSI* intron derived from GUSint (Vancanneyt et al., 1990) was placed under the control of the enhanced CaMV 35S promoter. The whole gene cassette was then transferred proximal to the *AscI*-site of the *AscI*/*PacI* flanked multiple cloning site of a shortened version of the pBIN19 vector kindly provided by G.J.A. Rouwendal. This vector lacks the left border sequence and contains only the RB sequence cloned proximal to the *PacI*-site. The dual selectable marker, the *codA* gene isolated from *Escherichia coli* by PCR and translationally fused to the *nptII* gene isolated from pBIN19 by PCR (Schaart et al., 2004), was introduced near the RB sequence. The *codA-nptII* hybrid gene was regulated by the CaMV 35S promoter. To allow visualization of transformation a *GUS* containing cassette under the control of the chrysanthemum *Rubisco* promoter (Outchkourov et al., 2003) was cloned into the multiple cloning site as an *AscI*/*PacI* fragment. This clone (Fig 1a) was designated PROGMO-GUS and used for the experiments described in Results section.

Potato Transformation and Regeneration

Solanum tuberosum cv. Désirée was vegetatively propagated from cuttings on MS medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose at 24°C with a 16h light-8h dark photoperiod under 5,000 lux intensity. Transgenic lines were obtained by leaf transformation according to Dietze et al. (1995) using the *Agrobacterium tumefaciens* strain AGL0 (Hood et al., 1986). Culture media contained 250 mg l⁻¹ cefotaxime for the elimination of *A. tumefaciens*, 50 mg l⁻¹ kanamycin for positive selection, and 150, 200, or 300 mg l⁻¹ 5-FC (Sigma) for negative selection.

Southern Hybridisation and PCR

DNA was isolated from in vitro grown plants according to the method of Shure et al. (1983).

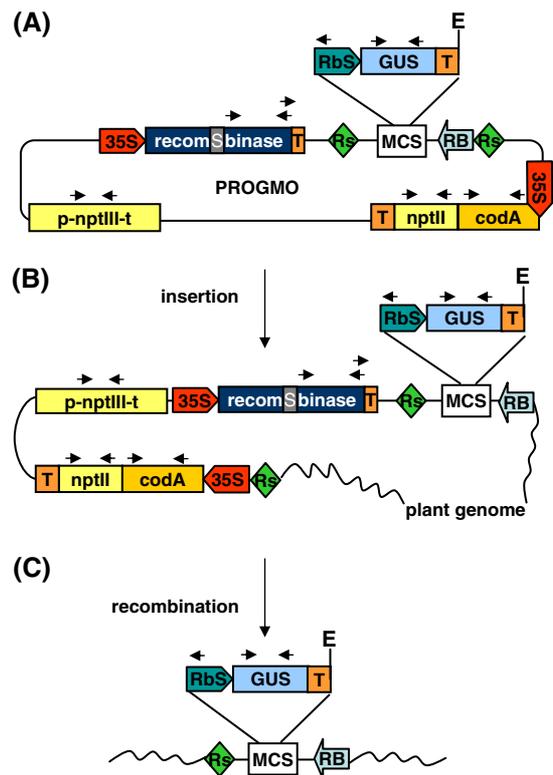


Fig. 1 Diagram depicting the 10-kb binary vector PROGMO and its derivative PROGMO-GUS (a). Insertion of the plasmid into the plant genome before (b) and after recombination (c). RB, right border; MCS, multicloning site for *EcoRI* (E), *XbaI*, *HindIII*, *PstI*, *SalI*, *AscI*, and *PacI*; T, terminator of *A. tumefaciens nos* gene; GUS, β -glucuronidase reporter gene; RbS; *Rubisco* promoter from chrysanthemum; Rs, recombination site; S, intron *ST-LSI* of *GUS* gene; 35S, CaMV 35S promoter; p-nptIII-t, neomycin phosphotransferase III gene with a bacterial promoter and terminator; nptIIcodA; hybrid gene for positive (*nptII*) and negative (*codA*) selection upon plant transformation. Arrows indicate the primers used for PCR. Drawing is not to scale

Twenty milligram DNA was digested with *EcoRI*, separated on 0.8% agarose gel, and blotted to Hybond N+ membrane. Hybridisation using radioactive DNA probe was carried out under stringent conditions as described by Dóczi et al. (2002).

For PCR analysis of transgenic lines the *codA* primers 5'-CGAATAACGCTTTACAAACAA-3', 5'-CGTTTGTAATCGATGGCTTG-3', the *nptII* primers 5'-TGGGCACAACAGACAAT CGGCTGC-3', 5'-TGCGAATCGGGAGCGGC GATACCG-3', the *nptIII* primers 5'-TCCACCT TATCGGCAATGAA-3', 5'-CGGCAGTGAG

AGCAGAGATA-3', the *rec* primers 5'-GCAA GGGAAGAAGTAGACGA-3', 5'-GGTGATG TTGTAGAAGC-3', the *rubrec* primers 5'-CCTG GCTGGTATCTCCTAAAG-3', 5'-CGCAGAA AGATTAGCATTGTTCG, and the *GUS* primers 5'-CTGTAGAAACCCCAACCCGTG-3', 5'-CA TTACGCTGCGATGGATCCC-3' were used.

Genome Walking

The BD GenomeWalker (Clontech) kit was used to isolate flanking sequences of transgenes. Genomic DNA of transgenic potato plants was digested with *DraI*, *EcoRV*, or *StuI*. The BD GenomeWalker adaptor was ligated to both ends of the genomic DNA fragments to create a library. The outer adaptor primer provided in the kit and the primer 5'-CCTGGGCTGGT ATCTCCTAAAG-3', specific for the 5' region of the *Rubisco* promoter, was used in the first PCR. The second PCR was performed with the inner adaptor primer and the nested *Rubisco* promoter-specific primer 5'-CCAGTGTACCCAAAGCG-TATCG-3'. Sequencing of the resulted fragments was performed in house by the DNA Sequencing Laboratory.

GUS Activity Assays

Histochemical and fluorimetric assays from leaves of in vitro grown potato plants were performed as described by Jefferson (1987). For fluorimetric activity measurements extracts were standardised based on protein concentration as determined by the method of Bradford (1976).

Results

Construction of the Binary Vector PROGMO

A binary vector, PROGMO (Figure 1a), was constructed on the basis of pRCNG used for strawberry transformation (Schaart et al., 2004). PROGMO contains the plant-adapted version of the *R* recombinase and the bi-functional translationally fused marker gene *codA-nptII*. In the vector plasmid pRCNG, the C-terminus of the *R* recombinase gene was fused to the ligand-binding

domain (LBD) of the rat glucocorticoid receptor to achieve induction of recombinase activity via dexametasone (DEX) treatment. It was found, however, that GUS-staining intensity and pattern for all tested lines was similar in untreated and treated plants indicating that recombination had already occurred before the start of DEX treatment (Schaart et al., 2004). Based on this finding, the *R* recombinase gene was cloned into the new vector, PROGMO, without the LBD domain, but with the CaMV 35S promoter to provide constitutive expression of the *R* gene. To avoid the synthesis of recombinase in *Agrobacterium*, the intron *ST-LSI* was placed into the *R* coding region. To facilitate easy cloning of genes of interest a multicloning site (MCS) was inserted downstream of the recombinase.

Previously constructed T-DNA transformation vectors utilise the right (RB) and left border (LB) sequences for T-strand production. The RB and adjacent 'overdrive' sequence is necessary for the initiation of T strand generation and transfer, whereas the LB seems to mediate the stop of the T strand (Tzfira et al., 2004 for a review). When this border is left out, the whole vector can be transferred to the plant cell. In contrast to normal T-DNA transformation vectors PROGMO carries only the RB. Furthermore, the backbone vector DNA has been reduced to the limited amount of DNA sequences, which are still necessary for proper maintenance, propagation and plant transformation. Thus the whole plasmid will be inserted as one large T-DNA into the plant genome. The RB is adjacent to the MCS. The recombinase recognition sites (*Rs*) are in direct repeat and surround the MCS-RB fragment. The *R* gene, the other part of the vector including the *nptIII* gene that is used for selection in bacteria, and the *codA-nptII* that is used for selection in plants are all outside the *Rs*-flanked fragment. When integration has taken place after linearization at the RB, the *R* gene, the selection marker genes and the rest of the backbone vector DNA flanked by *Rs* sites will be recombined and deleted from the genome when the recombinase enzyme is active.

Figure 1b shows integration of PROGMO into the plant genome. Recombinase-mediated site-specific excision will result in a marker- and

backbone-free transgenic line containing only the gene of interest flanked by a copy of *Rs* and RB (Fig 1c).

Generation of Marker- and Backbone-Free Potato Lines with Temporary Selection

Cultivar Désirée was used to test the efficiency of PROGMO transformation in potato. A *Rubisco* promoter-*GUS* construct was inserted into the MCS of PROGMO and the resulting plasmid, PROGMO-GUS, was used for *Agrobacterium*-mediated transformation of leaf explants. Rommens et al. (2004) have demonstrated that subsequently linking a positive selection for temporary marker gene expression in callus induction phase to a negative selection against marker gene integration during shoot regeneration increases the frequency of marker-free lines. Based on this finding, in the initial experiment, after transformation of Désirée leaf explants with PROGMO-GUS kanamycin selection was applied only during callus induction (seven days) while the negative selection by 150 mg l⁻¹ 5-FC (Rommens et al. 2004) was employed during the entire shoot regeneration period (3–4 weeks). Rooting of the plantlets was carried out in the presence of cefotaxime that suppresses *Agrobacterium* growth, but without selection for transgenic plants. Histochemical staining of 150 regenerants resulted in detection of GUS activity in the leaves of nine lines. This result indicated that a large number of non-transgenic shoots could start regeneration after we finished the callus induction phase with positive selection. To avoid this

problem longer periods of selection on kanamycin and higher concentrations of 5-FC in the medium were applied. Table 1 shows that the highest efficiency of selection for transgenic lines was achieved after 21 days of positive selection followed by negative selection in the presence of 300 mg l⁻¹ 5-FC in the medium. Using this mode of selection 6 GUS⁺ lines out of 14 regenerated shoots were obtained.

The GUS⁺ lines were further investigated by PCR. Using primer pairs specific for *codA*, *nptIII*, *nptIII*, the recombinase (*rec*) and the fragment connecting the recombinase and the *Rubisco* promoter (*rubrec*), PCR analysis revealed 16 marker- and backbone-free lines out of 35 GUS⁺ lines obtained (Table 1). The highest efficiency was detected after 21 days of positive selection followed by negative selection in the presence of 300 mg l⁻¹ 5-FC in the medium. This combination resulted in isolation of 4 marker- and backbone-free GUS⁺ transgenic lines out of 14 regenerated shoots, which means a frequency of 29%. As an example, Fig 2 shows six lines out of nine GUS⁺ lines that lack all the backbone and selection marker genes introduced by PROGMO-GUS except for *GUS*. In the other three lines a partial integration of PROGMO-GUS might have occurred resulting in the lack of the *codA-nptIII* selection marker, but with either the recombinase still present (TS4) or both recombinase and *nptIII* present (TS5 and 6). We suppose that due to the absence of one of the recombination sites recombination could not eventuate in these lines.

High copy number of transgene insertions may cause rearrangements and induce gene silencing.

Table 1 Efficiency of PROGMO transformation with temporary selection

5-FC mg l ⁻¹	Days on kanamycin								
	7			14			21		
	Reg ^a	GUS ^b	GUS ⁺ PCR ^c	Reg.	GUS ⁺	GUS ⁺ PCR ^c	Reg.	GUS ⁺	GUS ⁺ PCR ^c
150	150*	9	6	33	4	0	17	1	1
200	64	3	0	14	0	0	15	1	1
300	43	8	3	35	3	1	14	6	4

^aNumber of regenerants obtained from 15 leaf explants except of that labelled by asterisk in which case 40 explants were used.

^bNumber of regenerants showing GUS activity by histochemical staining

^cNumber of marker- and backbone-free regenerants showing GUS activity by histochemical staining. Presence of marker and backbone genes was tested by PCR (Fig 2)

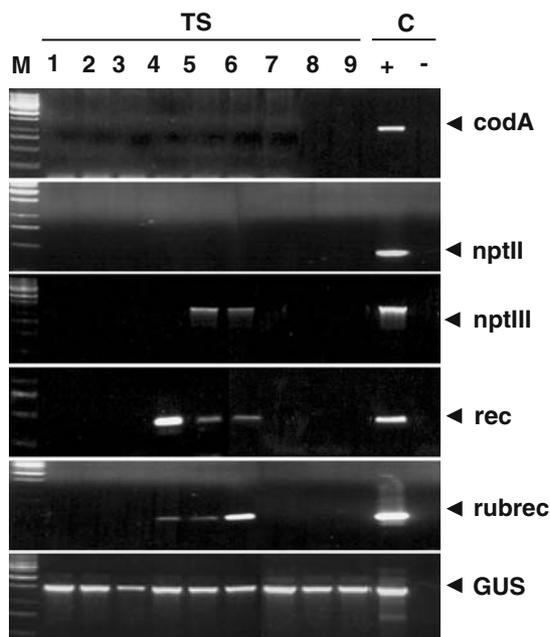


Fig. 2 Molecular analysis of putative marker-free *GUS* transgenic plants TS1–9 obtained by seven days positive selection followed by negative selection with 150 mg l^{-1} 5-FC in the shoot induction medium (Table 1). PCR analysis was carried out with primers specific for *codA*, *nptII*, *nptIII*, recombinase (*rec*), the region connecting the recombinase and the *Rubisco* promoter (*rubrec*), and for the *GUS* gene. PROGMO-*GUS* plasmid DNA was used as a positive (+) and non-transformed plant DNA (-) as a negative control (C). M, size marker

Therefore, isolation and application of single or low copy transgenic lines are desired for fundamental as well as for applied research. Copy number of the *GUS* gene in the six marker- and backbone-free lines was investigated by Southern hybridisation. The hybridisation pattern in Fig 3a demonstrates a low copy number *GUS* insertion in all six lines obtained. *EcoRI* has a single recognition site in the PROGMO-*GUS* construct (Fig 1a, b, c) *EcoRI* digested genomic DNA gave one hybridising band in one transgenic line (TS1) and two bands in five lines (TS2, 3, 7, 8, 9). Interestingly, the five lines with two bands carried a band of the same size of around 3.5 kb. To investigate the molecular background of the same size of fragments and to be certain about absence of vector backbone, a part of transgenic DNA and flanking genomic sequences were cloned by genome walking from TS2, 7, and 8. Nested PCR primers specific for the 5' region of the *Rubisco*

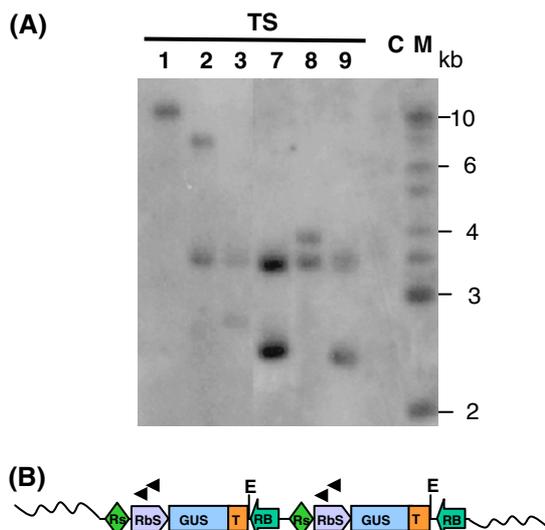


Fig. 3 (a) Southern blot analysis of marker-free *GUS* transgenic plants TS1, 2, 3, 7, 8, 9. DNA was digested with *EcoRI* and hybridised with *GUS* probe. PROGMO-*GUS* carries a single *EcoRI* site located between the RB and *GUS* sequences (Fig 1). C, non-transformed control plant; M, size marker. (b) Schematic overview of the repeat formation found by genome walking in lines TS2, 7 and 8. E, *EcoRI*; Arrows indicate the specific primers used for genome walking. Drawing is not to scale

promoter were designed to amplify the surroundings of transgenic DNA. The obtained PCR products were directly sequenced. Figure 3b gives an overview of the configuration found in each transformant tested. Sequence analysis showed two *GUS* genes in a tandem orientation without filler DNA between them. This configuration explains the existence of a *GUS*-hybridising *EcoRI* fragment of the same size of around 3.5 kb in the Southern blot (Fig 3a).

Quantitative data on *GUS* expression were obtained by fluorimetric assay. Very high activities ($20,000\text{--}33,000 \text{ pmol MU min}^{-1} \text{ mg}^{-1} \text{ protein}$) were detected in the lines obtained by PROGMO vector (Fig 4).

Discussion

We have succeeded in constructing an improved marker- and backbone-free transformation system based on plant-adapted R recombinase, *R*s sites, and a strongly in size reduced binary vector containing only one border (RB) sequence

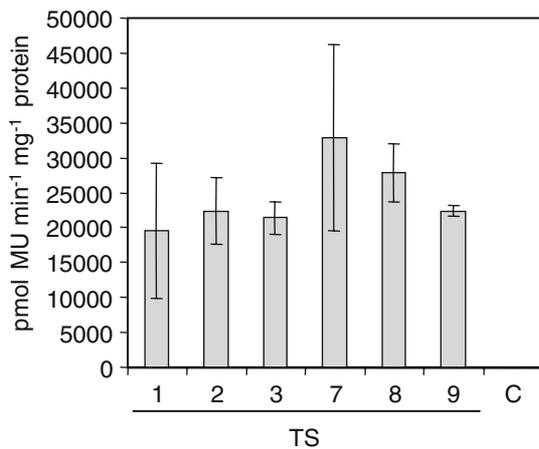


Fig. 4 GUS activity of marker-free *GUS* transgenic plants TS1, 2, 3, 7, 8, 9. C, non-transformed control plant. The bars show the average data obtained from two independent experiments. Error bars represent the standard error. Specific GUS activity is expressed as pmol MU min⁻¹ mg⁻¹ protein

(PROGMO vector). After integration of the complete binary vector the *R*s sites are orientated and located as such that the active recombinase enzyme will recombine and delete the recombinase gene, the markers and backbone sequence out of the plant genome resulting in marker- and backbone-free transgenic potato plants. In order to monitor and test the transformation and recombination events we also introduced a *codA-nptII* bi-functional selectable marker gene. Using 21 days of positive selection and 300 mg l⁻¹ 5-FC for negative selection we have obtained marker- and backbone-free transgenic potato plants with a frequency of 29% related to the number of regenerated shoots.

Our system differs from other *Agrobacterium*-mediated transformation approaches in using only the RB for T-DNA transfer. T-DNA border sequences are imperfect direct repeats. Horsch and Klee (1986) evidenced first for LBs occasionally functioning as RBs to initiate T-strand synthesis. Thus the transfer of backbone sequences reported for many plant species was possibly due to the initiation of T-strand production at the LB. In other events, this could be simply the result of failure to terminate a RB-initiated T-strand at LB. Formation of genetic chimeras have also been reported when LB-initiated T-DNA ligated back to a RB-initiated

T-strand (De Buck et al., 1999). The not correct initiating, processing and ending of the T-region during transformation of plant cells using *Agrobacterium*-mediated transformation method often results in integration of extra backbone DNA originating from the binary plasmid in the plant genome (de Vetten et al., 2003). By using only the RB and reducing the binary PROGMO vector backbone DNA, the complete vector will be integrated in the plant genome as one large T-DNA. After integration, the *R*s sites are orientated as such that the whole vector DNA (backbone DNA and selection marker genes, if used) is recombined out of the genome when the recombinase is active, except for the RB and the MCS in which the gene of interest is inserted.

Deletions and inversions using the *Z. rouxii* *R/R*s recombination system have been demonstrated in tobacco, *Arabidopsis* and rice (Onouchi et al., 1991; 1995; Toriyama et al., 2003). Here we have shown that the *R/R*s system is also active in potato. Interestingly, genome walking resulted in detection of two *GUS* transgenes in a tandem orientation in three marker- and backbone-free transgenic lines. Based on Southern hybridisation (Fig 3a) the same repeat formation may be present in five out of six lines tested. Thus our results further support the idea that T-DNA-transfer replication, originating at the T-DNA border, produces continuous strands via a rolling circle-type replication (Waters and Guiney, 1993).

All six marker- and backbone-free *GUS* transgenic lines obtained after temporary kanamycin selection on 5-FC containing plates showed high GUS activities driven by the *Rubisco* promoter (20,000–33,000 pmol MU min⁻¹ mg⁻¹ protein). Gittins et al. (2000) studied the ability of the *Rubisco* promoter in apple and got 12 transgenic lines with GUS activities ranging from 1,000–35,000 pmol MU min⁻¹ mg⁻¹ protein. Chitinase activity of 17 transgenic alfalfa plants containing the *Rubisco* promoter-*ech42* construct varied from 0 to 2,000 pmol MU min⁻¹ mg⁻¹ protein (Samac et al., 2004). A similar high range of GUS activities (1.8–109.0 pmol MU min⁻¹ g⁻¹ protein in ten lines) was reported in potato using the CaMV 35S promoter (Goldsbrough and Bevan, 1991). The considerable variations were explained by position effects. The PROGMO

transformation system may itself select for transgenic lines with high activities. Others showed before that the earliest detection of gene expression from T-DNA encoded genes started 18h after infection, peaked at 36h and declined over 4–10 days as the cells with only transiently expressed T-DNA encoded genes fail to become stably transformed (Jansen and Gardner, 1990; Narasimhulu et al., 1996). We incubated the leaf explants together with the *Agrobacterium* for 2 days and then applied kanamycin selection for 7, 14 and 21 days. Thus, by that time the recombinase has already been integrated into the plant genome. The 5-FC selects for loss of *codA-nptII*, which probably needs high recombinase activity that could be achieved only from certain chromosomal positions. After recombination the *GUS* remains at the same chromosomal position that may explain the high *GUS* activities of the marker- and backbone-free transgenic lines. The low copy number of insertions detected in the same lines is due to the higher probability of removing *codA* in low than in high copies.

The PROGMO vector approach is simple and might be widely applicable in the production of marker- and backbone-free transgenic plants for many crop species.

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