

# A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop

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It is generally thought that transformation of plant cells using *Agrobacterium tumefaciens* occurs at a very low frequency. Therefore, selection marker genes are used to identify the rare plants that have taken up foreign DNA. Genes encoding antibiotic and herbicide resistance are widely used for this purpose in plant transformation<sup>1,2</sup>. Over the past several years, consumer and environmental groups have expressed concern about the use of antibiotic- and herbicide-resistance genes from an ecological and food safety perspective. Although no scientific basis has been determined for these concerns, generating marker-free plants would certainly contribute to the public acceptance of transgenic crops. Several methods have been reported to create marker gene-free transformed plants, for example co-transformation, transposable elements, site-specific recombination, or intrachromosomal recombination<sup>3–9</sup>. Not only are most of these systems time-consuming and inefficient, but they are also employed on the assumption that isolation of transformants without a selective marker gene is not feasible<sup>10</sup>. Here we present a method that permits the identification of transgenic plants without the use of selectable markers. This strategy relies on the transformation of tissue explants or cells with a virulent *A. tumefaciens* strain and selection of transformed cells or shoots after PCR analysis. Incubation of potato explants with *A. tumefaciens* strain AGL0 resulted in transformed shoots at an efficiency of 1–5% of the harvested shoots, depending on the potato genotype used. Because this system does not require genetic segregation or site-specific DNA-deletion systems to remove marker genes, it may provide a reliable and efficient tool for generating transgenic plants for commercial use, especially in vegetatively propagated species like potato and cassava.

Potato variety Karnico was genetically transformed with a potato granule-bound starch synthase (GBSSI) antisense gene containing an additional, upstream inverted copy of its 5' region (pKGBA50mf-IR1.1, Fig. 1). The inclusion of a short repeated region of the target gene *GBSSI* within a transgenic construct resulted in a fourfold

**Table 1. Effect of strain of *Agrobacterium tumefaciens* on transformation efficiency of potato variety Karnico**

Experiment no.	PCR analysis		Phenotypic analysis	
	LBA4404	AGL0	LBA4404	AGL0
1	0/1,112 (0.0)	50/888 (5.6)	0/0 (0.0)	17/40 (42.5)
2	3/632 (0.5)	10/420 (2.4)	1/3 (33.3)	8/10 (80.0)
3	0/440 (0.0)	31/651 (4.8)	0/0 (0.0)	10/31 (32.3)
4	2/240 (0.8)	9/688 (1.3)	2/2 (100)	2/9 (22.2)
5	–	128/2,370 (5.4)	–	60/125 (48.0)
Total no. of shoots	5/2,424 (0.2)	228/5,017 (4.5)	3/5 (60.0)	97/215 (45.1)

<sup>a</sup>The number of PCR-positive shoots obtained from the total number of shoots tested (%).

<sup>b</sup>The number of amylose-free transformants obtained from the total number of transformants tested (%).

increase in the frequency of completely silenced transformants compared to a similar construct without the inverted repeat.

Construct pKGBA50mf-IR1.1 was transferred to *Agrobacterium tumefaciens* LBA4404 or *A. tumefaciens* AGL0. Strain AGL0 exhibits high transformation efficiency because it contains a DNA region originating from the virulence region of Ti plasmid pTiBo542 of the supervirulent *A. tumefaciens* A281 (ref. 11). In the course of five independent experiments, ~8,000 stem explants of potato variety Karnico were inoculated with either LBA4404 or AGL0 containing pKGBA50mf-IR1.1. Over a period of one to four months after inoculation, one or two regenerated shoots per explant were harvested and were allowed to grow on Murashige and Skoog medium<sup>12</sup>. After one or two weeks, leaf or stem material of eight independent shoots was harvested and pooled. DNA of these pools of shoots was isolated in 96-well microtiter plates and tested for the presence of transformants in the pools of shoots by PCR using primers P1 and P2 (Fig. 1). Subsequently, PCR analyses were conducted on DNA isolated from individual plants of the PCR-positive pools to select the transformants. After transformation with LBA4404, <0.2% of the harvested shoots were scored PCR-positive, whereas with AGL0 this percentage was on average 4.5% (Table 1). The frequency of PCR-positive transformants of the five independent transformation experiments with AGL0 ranged between 1.3% and 5.6%, whereas for LBA4404 this varied from 0% to 0.8%.

Marker-free transgenic plants were also obtained in genotypes that have a lower transformation efficiency than Karnico<sup>13</sup>. Three other commercially available varieties were selected, showing a percentage of PCR-positive shoots of 0.6% for var. Mercator, 1.7% for var. Kardal, and 2.4% for var. Seresta (see Supplementary Table 1 online).

To determine the frequency of PCR-positive shoots showing the desired amylose-free phenotype, shoots were grown *in vitro* under high-sucrose conditions to induce tubers. Developed microtubers were microscopically assessed for the presence of amylose in the starch granules. Of 220 PCR-positive Karnico transformants analyzed, 100 (45%) showed complete inhibition of GBSSI activity, as indicated by the red color of starch granules stained with iodine solution. The efficiency of expressing the desired phenotype of the PCR-positive Seresta transformants was similar to that of Karnico (44%).

These results indicate that it is feasible to select transformants without the use of marker genes by using a virulent *A. tumefaciens* strain and an optimized gene-silencing construct.

Because of the controversy regarding the incorporation of unwanted DNA in addition to the gene of interest, transformants should be isolated that do not possess backbone-vector DNA or

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**Figure 1.** Map of the T-DNA region of the selectable marker-free *GBSSI* gene-silencing vector pKGBA50mf-IR1.1. The gene cassette, controlled by the potato *GBSSI* promoter, contains the 1.1 kb 5' end of the *GBSSI* cDNA in an inverted-repeat configuration interrupted by the 1.3 kb 3' end of the *GBSSI* cDNA. Arrowheads indicate the position of primers on the T-DNA. Primers P1 and P2 were used for the detection of transformants. PCR products obtained with primer combinations PR1 + PR2 and PL1 + PL2 were used as probes in Southern blot hybridizations. RB, Right border; LB, left border;  $P_{GBSSI}$ , promoter of the potato *GBSSI* gene;  $T_{NOS}$ , polyadenylation sequence of nopaline synthase gene.

multiple T-DNA inserts. Integration of DNA beyond the borders into the genome of the host plants is reported to occur in 20–75% of transformed plants<sup>14,15</sup>. Selected marker-free amylose-free Karnico transformants were analyzed for presence of backbone-vector DNA by conducting five different PCR analyses using primers specific for five open reading frames representing 81% of the pBIN19 vector outside the T-DNA<sup>16</sup>. A total of 60 of the 99 tested amylose-free transformants were positive for one or more of the PCR reactions, whereas 39 transformants were negative for all five vector DNA fragments. Southern blot hybridization with the pBIN19 backbone-vector DNA confirmed the absence of integrated non-T-DNA sequences in all clones tested (data not shown). Of the 12 amylose-free Seresta transformants tested, 11 were free of vector DNA sequences.

The 39 vector DNA-free Karnico transformants were further analyzed by Southern blot hybridization to determine the number of T-DNA insertions. Hybridizations were carried out using a nopaline synthase (NOS) terminator probe close to the left border of the T-DNA, and a probe close to the right border (Fig. 1). These analyses showed that most (28) transformants contained three or less T-DNA insertions (Fig. 2A). Moreover, of the 39 transformants analyzed, ten contained a single T-DNA insertion as determined with the two hybridization probes. This suggests that it is feasible to obtain marker-free potato transformants free of backbone-vector DNA containing only one T-DNA insertion.

During the staining of one tuber per independent transformant, no sectorial staining was observed. All tubers were either completely red or completely blue. Iodine staining of >20 tubers each of 60 independent transformants showed only one transformant with tubers displaying red and blue sectors (Fig. 2B). These observations indicate that chimerism is occurring but at a very low frequency.

To test whether PCR selection of transformants is also feasible for other vegetatively propagated crops, we infected friable

embryogenic callus (FEC) derived from cassava TMS60444 with the *A. tumefaciens* strain AGL1 containing the firefly luciferase gene as gene of interest. *Agrobacterium tumefaciens* strain AGL1 is similar to AGL0, but *recA*<sup>-</sup> (ref. 17). Three independent transformation experiments were carried out. After transformation, the FEC lines were cultured for one week in liquid medium before spreading them out finely on solid proliferation medium. Four weeks later the presence of colonies was observed; these were subsequently screened for luciferase activity. The percentage of luciferase-positive colonies was on average 4.7% (Table 2) ranging between 0.6% and 9.5% for the three experiments. The positive colonies were cultured for the regeneration of somatic embryos. Of the 103 luciferase-positive colonies obtained from the three transformation experiments, 43 colonies produced luciferase-positive somatic embryos, indicating that of the 2,193 colonies obtained, 2% could form luciferase-positive somatic embryos. All of 12 luciferase-positive somatic embryos that were cultured for plant regeneration resulted in luciferase-positive plants. These results, in combination with Southern blot experiments conducted earlier<sup>18</sup>, indicate that they are stable transformants. Based on these transformation efficiencies, we expect that PCR-based selection of transformants is also applicable to cassava.

In conclusion, we demonstrate that the use of a selectable marker gene can be avoided in the transformation process of plants. Using a PCR detection method, it is possible to distinguish plants that carry a transgene from nontransformed plants. With a highly efficient silencing construct and a virulent *A. tumefaciens* strain, and depending on the potato genotype used, >2% of the harvested shoots were transgenic and showed a complete silencing of the *GBSSI* gene resulting in an amylose-free phenotype. Compared to other methods reported to create marker gene-free transformed genotypes, such as co-transformation and subsequent crossing out of the marker gene<sup>3</sup>, use of a *Cre/lox* or other recombinase systems to remove the marker gene after transformation<sup>4,7,8</sup>, or a transposase-driven removal of the marker gene<sup>5,6</sup>, our method has several advantages.

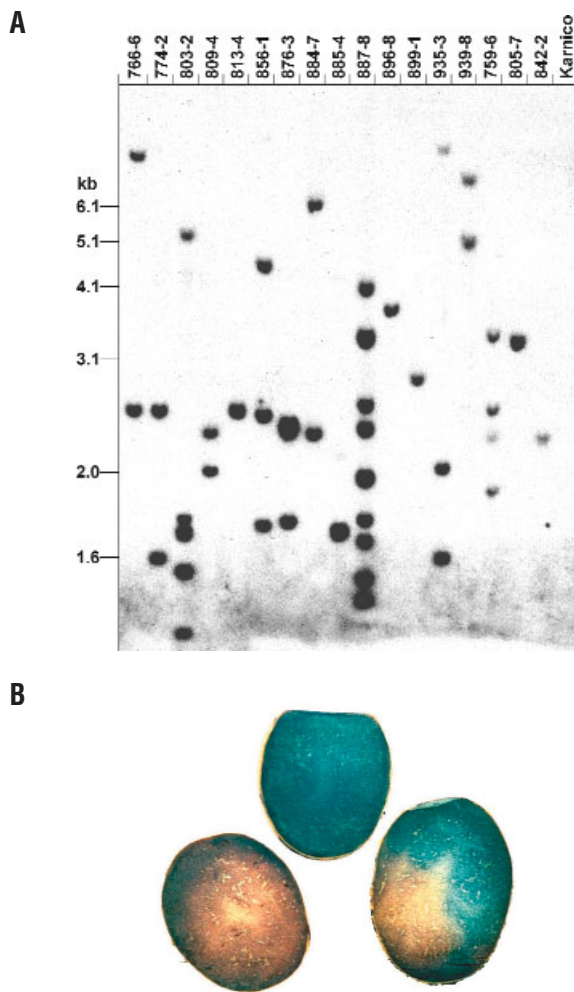
Our method does not require genetic segregation, which in the case of many cross-pollinating, vegetatively propagated crops like potato would require many years of breeding to find a genotype with suitable field performance. Vegetatively propagated crops such as potato, grapevine, strawberry, banana, and cassava are highly heterozygous, requiring vegetative propagation to maintain the elite genotype. Attempts have been made to adapt transposon and recombinase methods to vegetatively propagated crops<sup>4-6,8</sup>. These methods are based either on the failure of an excised transposon to reinsert back into the genome or on transient introduction

**Table 2. Transformation efficiency of cassava**

Experiment no.	Luc <sup>+</sup> colonies <sup>a</sup>	Luc <sup>+</sup> somatic embryos <sup>b</sup>
1	3/479 (0.6)	2/479 (0.4)
2	52/548 (9.5)	26/548 (4.7)
3	48/1,166 (4.1)	15/1,166 (1.3)
Total	103/2,193 (4.7)	43/2,193 (2.0)

<sup>a</sup>The number of luciferase-positive colonies obtained from the total number of colonies tested (%).

<sup>b</sup>The number of luciferase-positive somatic embryos obtained from the total number of colonies tested (%).



**Figure 2.** Molecular and phenotypic analysis of the marker-free transformants. (A) Southern blot analysis of DNA isolated from marker-free, vector-free, and amylose-free potato transformants. Genomic DNA was isolated from 17 transformants and 1 untransformed control (Karnico), digested with *HindIII*, and probed with the NOS terminator probe. (B) Longitudinal section of potato tubers after iodine staining of an amylose-free potato transformant (left), an amylose-containing transformant (middle), and a chimeric transformant (right). Blue staining indicates the presence of amylose and red its absence.

of the recombinase gene to delete the marker gene. Both variants are not very efficient, however, and because another cycle of regeneration is required the obtained plants are more prone to somaclonal variation. A second advantage of our method is that because no selectable marker is used, the system does not require the time-consuming step of analysis for reinsertion of transposed or recombined fragments into other genomic positions. Third, genetic chimeras are obtained only sporadically (<2% are chimeric), whereas with recombination systems the percentage may be higher<sup>4,8</sup>. And finally, our method is efficient enough to generate hundreds of independent transformation events, so that one may identify a clone that contains the gene of interest, is free of undesirable vector sequences, and is expected to have the same agronomic performance as the donor parent. Other methods adapted to vegetatively propagated crops like transposon excision and intrachromosomal recombination<sup>5,9</sup> are not very efficient, need successive rounds of screenings, and are therefore not applicable to generate hundreds of marker-free transformants.

## Experimental protocol

**Constructs.** The antisense *GBSSI* construct pKGBA50 has been described<sup>19</sup>. It is a pBIN19-derived construct that contains the 2.4 kb *GBSSI* cDNA in antisense orientation behind the *GBSSI* promoter, in addition to selectable marker gene *NptII*. Binary vector pKGBA50 was digested with enzymes *PmeI* and *Clal* to remove the *NptII* gene. The *Clal* sticky end was made blunt-ended by Klenow polymerase treatment, after which the vector DNA was circularized by blunt-end ligation using T4 DNA ligase. This resulted in vector pKGBA50mf (marker gene-free). The *GBSSI* cDNA 1.1 kb 5' portion was cloned as an *EcoRI* fragment into vector pMTL25, resulting in plasmid pMTL1.1. Vector pKGBA50mf was digested with *SaII*, and the 1.2 kb *SaII* fragment of pMTL1.1 was cloned into this vector. Both possible orientations of the 1.2 kb fragment were obtained. Binary vector pKGBA50mf-IR1.1 contains the 1.1 kb fragment of *GBSSI* in an inverted-repeat orientation.

All constructs were transformed into *Escherichia coli* DH5 $\alpha$  (Invitrogen, Breda, The Netherlands). The binary vector pKGBA50mf-IR1.1 was transformed into *A. tumefaciens* strain LBA4404 (pAL4404)<sup>20</sup> and strain AGL0<sup>17</sup> by triparental mating.

**Transformation of potato and selection of transformants.** Internodal cuttings from *in vitro*-grown plants of potato were used for transformation by *A. tumefaciens* co-cultivation<sup>21</sup>. Transformation was carried out with pKGBA50mf-IR1.1 in *A. tumefaciens* LBA4404 or *A. tumefaciens* AGL0. No selection was conducted. After four weeks the first shoots were harvested, and harvesting of shoots continued for about three months. No more than two independent regenerants per stem explant were harvested. Shoots were allowed to grow on MS30 medium (Murashige and Skoog medium<sup>12</sup> with 30 g/L sucrose). After one to two weeks, leaf or stem material of eight independent shoots was harvested and pooled. DNA of these pools of shoots was isolated in 96-well microtiter plates using the Magnesil genomic DNA isolation kit purchased from Promega (Madison, WI).

PCR analyses were done on DNA isolated from the pools of regenerants with the primers P1 5'-GCACCCCAGGCTTTACTT-3' (annealing 5' to the multiple cloning site of the pBIN19 vector) and P2 5'-TACCGTACACTTGACATTC-3' (annealing to the potato *GBSSI* promoter sequence), to check for the presence of transformants. The PCR cycles used for detection of the transgene were, 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 55 °C, 45 s at 72 °C, and finally, 7 min at 72 °C. PCR products were fractionated by electrophoresis in a 1.2% (wt/vol) agarose gel for detection of the 644 bp amplification fragment. Of the PCR-positive pools, leaf and stem material of individual shoots was harvested in 96-well microtiter plates, genomic DNA was isolated, and PCR analysis was done as described earlier.

***In vitro* tuberization.** PCR-positive shoots were grown on 50 ml MS30 medium. After three to four weeks, 20 ml of a liquid medium was added, consisting of KI medium (knol inducerend, or tuber-inducing medium; Duchefa, Haarlem, the Netherlands) with 325 g/l sucrose and 1.75 g/l CCC (chlorocholine chloride, or cycocel). The pots were placed in a dark growth chamber at a temperature of 18 °C. After two to four weeks, microtubers had developed on most shoots.

**Starch staining.** Microtubers were cut and stained with a 1:2 LUGOL-H<sub>2</sub>O solution (LUGOL is a 5% (wt/vol) iodine and 10% (wt/vol) potassium iodide solution (VWR International B.V., Amsterdam, The Netherlands)), to assess the presence of amylose in the starch granules. Staining of the starch granules was inspected with a binocular microscope (Olympus CH2; Paes, Zoeterwoude, The Netherlands).

**DNA analyses.** DNA was isolated from greenhouse-grown plants by the CTAB DNA isolation protocol<sup>22</sup>. PCRs were done with primers for the *nptIII*, *trfA*, *insB*, *oriV*, and *tetR* DNA sequences in pBIN19 to study the presence of backbone-vector DNA in the transformants<sup>16</sup>. A 4  $\mu$ g aliquot of DNA of the transformants was digested with *HindIII*, fragments were separated by gel electrophoresis, and Southern blots were made on Hybond N<sup>+</sup> membrane (Amersham Biosciences, Roosendaal, The Netherlands). Blots were hybridized with a NOS terminator probe or a right border probe, to check the number of T-DNA insertions. Primers used for PCR amplification of the NOS terminator probe:

PL1: 5'-ATGAGATGGGTTTTATGAT-3'

PL2: 5'-TTGAGTGTGTCCAGTTTG-3'

Primers used for PCR amplification of the right border probe:

PR1: 5'-TGCTAATGGTAATGGTGCTA-3'

PR2: 5'-CGGAAGCATAAAGTGTAAG-3'

To check for the presence or absence of backbone-vector DNA, a Southern blot was hybridized with the 8.0 kb *Bgl*II fragment of pBIN19, which contains 93% of the non-T-DNA sequence.

**Cassava transformation.** FEC of the cassava genotype TMS60444 was transformed with *A. tumefaciens* strain AGL1 (ref. 17) containing the firefly luciferase gene. Transformation and regeneration were as described before<sup>18,23</sup>.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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#### Competing interests statement

The authors declare that they have no competing financial interests.

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