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ORIGINAL PAPER

Genetic transformation of apple (*Malus* x *domestica*) without use of a selectable marker gene

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Abstract Selectable marker genes are widely used for the efficient transformation of crop plants. In most cases, antibiotic or herbicide resistance marker genes are preferred because they tend to be most efficient. Due mainly to consumer and grower concerns, considerable effort is being put into developing strategies (site-specific recombination, homologous recombination, transposition, and cotransformation) to eliminate the marker gene from the nuclear or chloroplast genome after selection. For the commercialization of genetically transformed plants, use of a completely marker-free technology would be desirable, since there would be no involvement of antibiotic resistance genes or other marker genes with negative connotations for the public. With this goal in mind, a technique for apple transformation was developed without use of any selectable marker. Transformation of the apple genotype "M.26" with the constructs *pPin2Att35SGUSintron* and *pPin2MpNPR1* was achieved. In different experiments, 22.0–25.4% of regenerants showed integration of the gene of interest. Southern analysis in some transformed lines confirmed the integration of one copy of the gene. Some of these transformed lines have been propagated and used to determine the uniformity of transformed tissues in the plantlets. The majority of the lines are uniformly transformed plants, although some lines are chimeric, as also occurs with the conventional transformation procedure using a selectable marker gene. A second genotype of apple, "Galaxy," was also transformed with the same constructs, with a transformation efficiency of 13%.

Keywords Genetically engineered · Markerless DNA transformation technology · Clean transformation

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Introduction

Improvement of trees by conventional breeding is constrained by their long juvenile periods, by loss of desired genetic combination, and by the complex reproductive characteristics of most of these species, including self-incompatibility and a high degree of heterozygosity. Genetic transformation offers an attractive alternative to breeding because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background (Pena and Séguin 2001). Genetic transformation of plants usually requires the inclusion of marker genes that enable the selection of transformed plant cells and tissues.

Although approximately 50 marker genes used for transgenic plant research or crop development have been assessed for efficiency, biosafety, scientific applications, and commercialization (Miki and McHugh 2004), only three selectable marker genes were used in more than 90%

of the scientific reports (Miki and McHugh 2004). These three genes are for resistance to the antibiotics kanamycin and hygromycin and to the herbicide phosphinothricin. The presence of these selectable marker genes in the genetically engineered (GE) plants has raised concerns regarding their potential transfer to other organisms and their safety (Flavell et al. 1992; Fuchs et al. 1993). In the case of antibiotic resistance markers, there is a fear that their presence in genetically modified crops could lead to an increase in antibiotic-resistant bacterial strains. In the case of herbicide resistance markers, there is concern that the markers will contribute to the creation of new aggressive herbicide-resistant weeds.

The avoidance of antibiotic or herbicide resistance markers in GE plants has been encouraged. Several positive (promoting the growth of transformed tissues) or negative (causing death of the transformed tissue) selection systems have been developed in recent years. These include systems based on nonmetabolizable agents such as xylulose (Haldrup et al. 1998a, b), mannose (Joersbo et al. 1998, 1999; Negretto et al. 2000; Reed et al. 2001; Boscariol et al. 2003; He et al. 2004), 2-deoxyglucose (Kunze et al. 2001), and benzyladenine-*N*-3-glucuronide (Joersbo and Okkels 1996) or based on the promotion of plant regeneration without the use of a selective agent, such as isopentenyl transferase (Kunkel et al. 1999; Endo et al. 2001; Zuo et al. 2002). Transgenic apple can be produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Examples are phosphomannose isomerase (Flachowsky et al. 2004; Zhu et al. 2004; Degenhardt et al. 2006; Szankowski and Degenhardt 2006) and Vr-ERE (Chevreau et al. 2006). Only Degenhardt et al. (2006) reported the regeneration of transgenic apple lines using the *pmi* gene as selectable marker with a rate of transformation from 1% to 24%. The other report showed the expression of the reporter gene in the transformed leaves, but they did not regenerate into plants.

For the commercialization of transgenic plants, it would simplify the regulatory process and improve consumer acceptance to remove gene sequences that are not serving a purpose in the final plant cultivars (Scutt et al. 2002; Miki and McHugh 2004). Although a number of strategies have been described for generating marker-free transgenic plants, all are more difficult to implement or less efficient than procedures that leave the marker genes in the plant. Of these different strategies, cotransformation of the genes of interest and the selectable marker genes on separate plasmids (Ebinuma et al. 2001; Miki and McHugh 2004) followed by rounds of segregation to create marker-free plants is an attractive alternative. However, this approach is not suitable for vegetatively propagated plants such as apple and pear. For these species, the use of transposons [such as the *Ac*/Ds transposable element system (Goldsbrough et al. 1993; Cotsaftis et al. 2002) or *ipt*-type multi-autotransformation vector system (Ebinuma et al. 1997a, b; Ballester et al. 2007)] or homologous recombination [such as the cre-lox system (Gleave et al. 1999; Zuo et al. 2001; Cuellar et al. 2006; Luo et al. 2007) and *FLP-FRT* system (Kilby et al. 1995; Luo et al. 2007)] to eliminate the marker gene may work at very low efficiency in apple. That was the case when Schaart et al. (2004) were emphasizing systems in which the marker genes are eliminated efficiently soon after transformation by using the cre-lox system. They were able to produce some transgenic Elstar containing no selectable marker, but with a low efficiency of transformation.

Here, we report the transformation of apple tissue without any selectable marker in the binary vector. Success of the procedure is dependent on the use of a highly efficient transformation system (Borejsza-Wysocka et al. 1999; Norelli et al. 1996).

Materials and methods

Plasmid constructs

Two binary expression vectors, pPin2Att.35SGUSint+.nptII− (1) and pPin2MpNPR1.GUS−.nptII− (2) were used in the present study (Fig. 1a, b, respectively). pWiAtt.35SGUSint+.nptII− (Norelli et al. 1994) contained a *npt*II-based expression cassette as a selectable marker, an *attacin* gene driven by the Pin2 promoter, and a *uidA* intron expression cassette driven by the 35S promoter adjacent to the right border of the T-DNA. pBinMpNPR1 (Malnoy et al. 2007) contained an *nptII*-based expression cassette as a selectable marker and the *Malus x domestica* Mp-NPR1 gene under the control of the pPin2 promoter. The *nptII*-based expression cassette of each of these two binary vectors was eliminated using the restriction enzymes, *Asc*I/*Bss*hII and *Nhe*I/*Cla*I, respectively, to produce 1 and 2. These binary vectors were introduced by electroporation into the supervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) containing the plasmid pCH32.

Plant material and transformation

The apple cultivar rootstock M.26 was chosen for this study because it can be genetically transformed at high efficiency (Borejsza-Wysocka et al. 1999; Norelli et al. 1996). Leaf segments were excised from in vitro grown shoots of this cultivar 3 weeks after subculturing. Transformation experiments were carried out as previously reported (Borejsza-Wysocka et al. 1999; Norelli et al. 1996) using *A. tumefaciens* strain EHA105 (Hood et al. 1993) containing

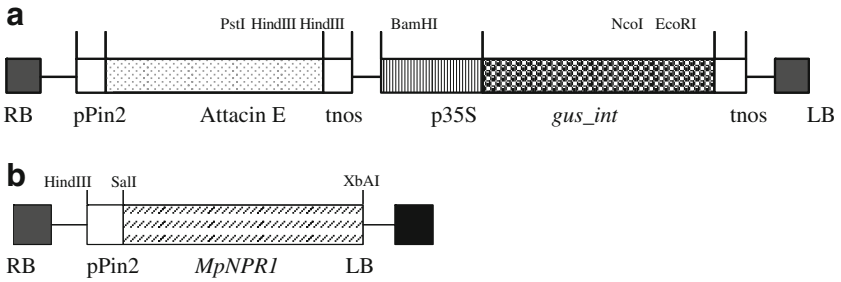

Fig. 1 Schematic diagram of the T-DNA region from the binary vectors pPin2att.35SGUSint+.nptII− (**a**) and pPin2MpNPR1.GUS−.nptII− (**b**). *RB* and *LB*, T-DNA right and left border sequences, respectively; *tnos*, nopaline synthase gene terminator, respectively; *p35S*, cauliflower mosaic virus 35S promoter; *pin2*, wound-inducible proteinase inhibitor II promoter from potato; *attacin E gene*; *MpNPR1*, *NPR1* gene from *Malus*, *gus_int*, intron-containing β-glucuronidase gene

pPin2iAtt.35SGUSint+.nptII− or pPin2MpNPR1.GUS−.nptII− binary vectors. The cocultivation and regeneration media contained no selection agents. The regeneration media contains cefotaxim to inhibit the growth of *Agrobacterium.* All regenerants were transferred to the M.26 proliferation medium without selection agents. DNA was isolated from the youngest leaf of putative transgenics and nontransformed control plants was isolated using the "Nucleon phytopure plant and fungal DNA extraction kits" protocol (Amersham, Piscataway, NJ, USA), and the polymerase chain reaction (PCR) procedure was as described by Bolar et al. (1999). Specific primers were designed to check for the presence of genes of interest (Table 1). Nontransgenic M.26 and transgenic clones were propagated in vitro (Norelli et al. 1988). The efficiency of transformation was calculated as the percentage of inoculated leaf segment explants that were determined to be transgenic by PCR.

Histochemical GUS assay

Transgenic in vitro shoots of apple were histochemically assayed for beta-glucuronidase (GUS) activity, using the histochemical staining procedure described by Jefferson et al. (1987) with some modifications. Samples were incubated overnight at 37°C in a solution containing 3 mM X-Glu, 4 mM potassium ferricyanide, 0.05 mM potassium ferrocyanide, 10 mM EDTA, and phosphate buffer (0.02 M, pH 7.2). Clearing was achieved using 70 % (*v*/*v*) ethanol.

Semiquantitative reverse transcription PCR analysis

Reverse transcription (RT) was conducted as described by Promega (Madison, WI, USA) with 1 μg of total RNA, extracted from 0.5 g of young leaves excised from greenhouse shoots according to the kit from Agilent (Wilmington, DE, USA). In order to evaluate relative differences in cDNAs between transgenic clones, comparative kinetic analysis was conducted by PCR using a procedure described by Malnoy et al. (2007). The quantification was done after 15 PCR cycles with −RT controls, in which the RT enzyme was omitted from the RT reaction in order to show that no genomic DNA remained in the samples after DNAse treatment. The primers used in this study are reported in Table 3.

Southern blot

DNA was extracted from the leaf tissue of nontransformed and putative transgenic plants according to the procedure of the nucleon extraction and purification kit (Amersham). Southern analysis was performed using standard procedures

Table 1 Primer sequences used for polymerase chain reaction

Target gene		Specific *Malus* spp. primer sequence	Tm °C
EF 1-α	F	ATTGTGGTCATTGGTCATTGT	54
	R	CCAATCTTGTAGACATCCTG	
NPR1	F	TCTTCAGTCGACATGGCTCATTCAGCCGAACCATCATCC	58
	R	CCACTAGTCAGTAAACCTCCGAAGGCTTATTAGATGC	
Attacin	F	CGGGATCCGCACGGAGCCCTTACGCTCA	55
	R	CCAAGCTTTCAGAAATATTTAGAAAGTGAGAAT	
GUS	F	GACGTAAGGGATGACGCACAAT	60
	R	CAGCAGCAGTTTCATCAATCA	
VirG	F	GAGTACTCTCTTCCGCGACG	56
	R	TTGGAATATCAGACTTTGCCG	

from Sambrook et al. (1989). Genomic DNA was digested with *Hin*dIII or *Eco*RI, electrophoretically separated on 0.8% agarose gel, and transferred to a nylon membrane (Hybond N, GE Healthcare Life Sciences, Piscataway, NJ, USA). Southern blotting was done with full sequence pin2, attacin, or *nptII* probes labeled with digoxigenin-11-dUTP, following the procedure of the DIG DNA labeling and detection kit (Roche, Indianapolis, IN, USA). The hybridization was done at 55°C.

Results

Apple transformation and selection of putative markerless regenerants

After 3 days of cocultivation with the binary vectors, pPin2Att.35SGUSint+.nptII− and pPin2MpNPR1.GUS−.nptII− (Fig. 1), leaf explants of M.26 and Galaxy were cultured on their appropriate regeneration media, without kanamycin, for 6 weeks. Each regenerated shoot was then collected and transferred to kanamycin-free proliferation media. For each transformation, between 1,200 and 1,800 regenerants were collected and grown (Table 1). Some of the regenerants showing normal growth were tested by PCR or GUS histochemical assays to screen for the integration of the T-DNA. For M.26, the four transformations conducted with two different binary vectors using the markerless DNA transformation technology (MDTT) showed a similar efficiency of transformation of approximately 24% (Table 1). In Galaxy, the efficiency of transformation was lower than for M.26 but still substantial with a mean transformation efficiency of 13%.

In both genotypes, GUS histochemical assays indicated nonuniform expression of the GUS protein in the putative transformants obtained by MDTT (Fig. 2). Due to the fact that the GUS gene construct contained an intron to prevent its expression in *A. tumefaciens*, the expression of the GUS protein in the putative transformants is due to the expression of the *gus* gene in the apple genome, which was confirmed by checking for the presence of the transgene by PCR. Additionally, the GUS-positive results were not "false" positives because no GUS staining was observed in the nontransformed control plants or in the pPin2MpNPR1.GUS−.nptII− transformants (Table 1). We observed a distribution of intense blue spots along the stem of the putative MDTT transformants compared to some transgenic lines obtained by conventional transformation

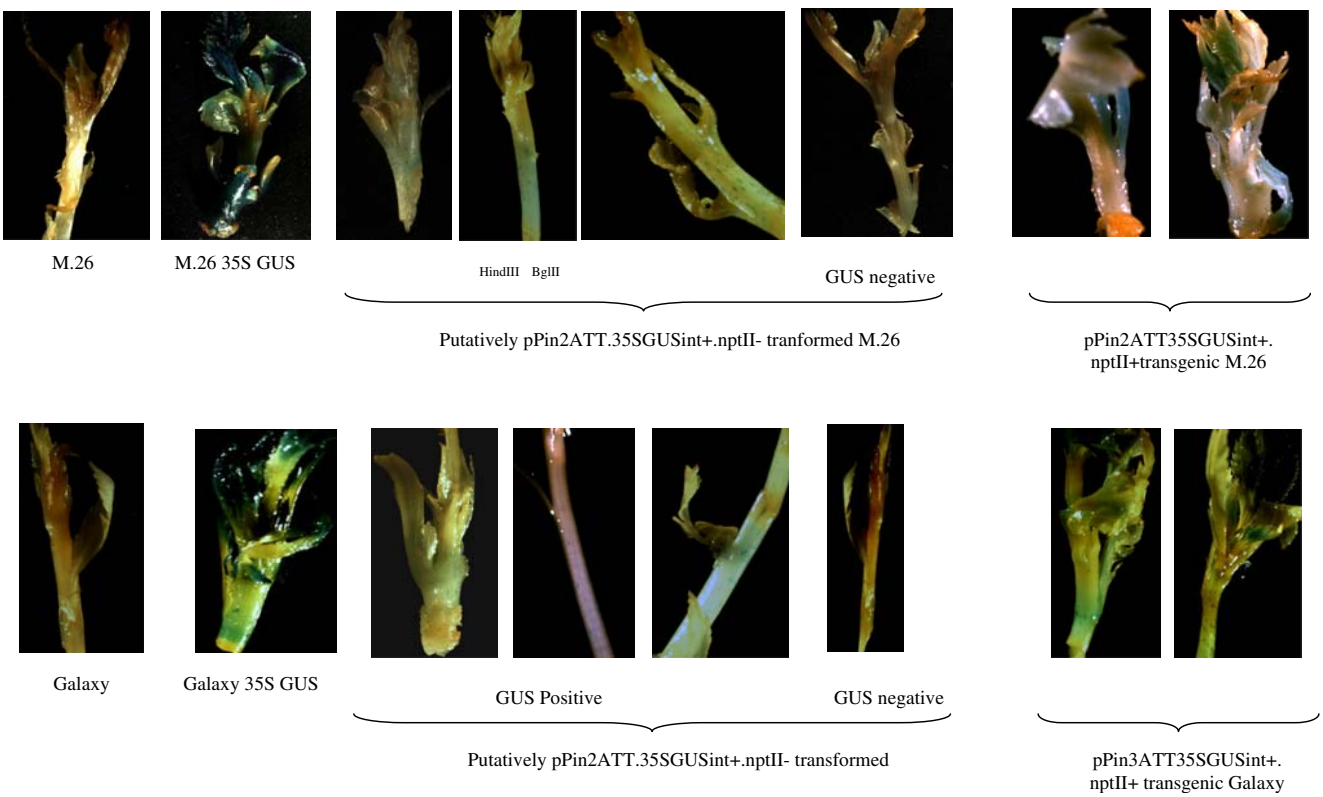

Fig. 2 Histochemical GUS assays on putatively pPin2ATT.35SGUSint+.nptII− transformed M.26 and Galaxy obtained following the markerless DNA transformation technology (*left*) or the conventional transformation procedure with *nptII* marker gene (*right*)

using kanamycin selection with a binary vector pPin2Att.35SGUSint+.nptII−. This pattern of GUS expression could be attributed to silencing of the gene or the fact that the markerless transformants are chimeric (Ko et al. 1998). A similar pattern of GUS expression was observed in regenerants obtained with the binary vector pPin2Att.35SGUSint+.nptII+ harboring the *nptII* selectable marker cassette (Fig. 2). This indicates that the nonuniform GUS expression could be due to the staining procedure, to silencing, or to chimeric regenerants. To address this question, several MDTT-transformed lines of M.26 and Galaxy expressing GUS (R1 generation) were selected and subjected to a regeneration process. The selected transgenic lines were micropropagated, and leaves from these lines were wounded to regenerate new shoots (R2 generation). Shoots from the R2 generation were transferred to a proliferation medium and tested for expression of the GUS protein by the histochemical assay (Table 2 and Fig. 3). Between 65% and 80% of the MDTT R2 M.26 and Galaxy regenerants showed some level of GUS activity. However, the number of areas stained in the R2 M.26 and Galaxy generation was higher than in the original R1 MDTT transformants (data not shown). The proportion of GUS-positive MDTT R2 M.26 and Galaxy plants was lower than that of transgenics obtain by classical transformation procedures using kanamycin selection (chi-squared 21.83 $P<0.0001$ for M.26 and chi-squared 72.21 $P<0.0001$ for Galaxy). When highly stained MDTT R2 regenerants were subjected to a second round of regeneration, the proportion of MDTT R3 regenerants with GUS staining increased (chi-squared 17.59 $P<0.0001$ for M.26 and chi-squared 73.89 $P<0.0001$ for Galaxy) compared to the R2 generation and became similar to that of transgenic lines produced by classical transformation procedures using

Table 2 Percentage of transformed lines with GUS staining after several regeneration episodes

		R2 generation				R3 generation			
Cultivars	Constructions	Lines	No. of regenerants tested	No. of lines GUS positive	Rate %	Lines	No. of regenerants tested	No. of lines GUS positive	Rate %
		First regeneration				Second regeneration			
M26	pBI121 (CaMV35S GUS int+.nptII +)	625	348	341	98.1				
		638	168	161	95.8				
	pPin2tAtt.35SGUS int+.nptII+	679	326	276	84.7				
		658	247	204	83.0				
	pPin2tAtt.35SGUS int+.nptII−	162	85	67	78.8				
		468	120	83	69.1	468-56	349	284	81.4
		622	103	79	76.7	622-22	267	220	82.4
		850	99	79	79.8	850-41	352	303	86.1
		870	138	101	73.2	870-51	272	243	89.3
		1292	144	108	75.0	1292-108	116	93	80.2
Galaxy	pBI121 (CaMV35S GUS int+.nptII +)	171	429	412	96.0				
		172	321	315	98.1				
	pPin2tAtt.35SGUS int+. nptII−	18	70	49	70.0				
		74	52	38	73.0				
		75	195	144	73.8	75-21	245	195	79.9
		156	76	51	67.1	156-3	256	213	83.3
		183	130	84	64.6	183-44	178	155	87.1
		186	97	62	63.9	186-78	276	223	80.3
		191	192	130	67.7	191-32	198	167	84.3
		193	136	91	66.9	193-87	254	212	83.5

kanamycin selection (chi-squared 0.37 $P<0.5393$ for M.26 and chi-squared 0 $P<0.9975$ for Galaxy; Table 2). Some of the plants from the MDTT R3 generation had almost uniform staining (Fig. 3, right picture).

Molecular characterization of *attacin E* and *GUS* gene expression in the MDTT lines

To demonstrate expression of the different genes in the putative MDTT-transformed lines, mRNA levels were determined in MDTT transgenic lines and compared to those of nontransformed M.26 plants and transgenic lines obtained by classical transformation using kanamycin selection and with the pPin2Att.35SGUSint+.nptII− binary vector. The selected lines displayed different levels of attacin and GUS mRNA, as revealed by semiquantitative RT-PCR (Fig. 4). No attacin or GUS mRNA signal was detected in the nontransformed control M.26 or in two MDTT lines that did not have an integrated gene of interest. No, *nptII* mRNA signal could be detected in the MDTT lines and control M.26, whereas a signal was detected in the transgenics transformed with an *nptII* vector by classical methods. The absence of the *nptII* gene was confirmed in the MDTT lines by Southern blot (data not shown), while the integration of only one copy of the *attacin E* or *GUS* gene was detected in most of the MDTT lines (Fig. 5). Only two MDTT lines out of ten showed insertion of two copies of the *attacin E* and *GUS* genes.

Similar results were obtained for the Galaxy MDTT and transgenic lines expressing the *attacin E* and *GUS* genes (data not shown).

To determine if regenerants were contaminated with *Agrobacterium*, some R1 regenerants obtained by MDTT and 25 obtained by classical transformation procedures were screened by PCR using primers designed for *virG* of *A. tumefaciens* (Table 3). Only in 1% to 3% of the MDTT-derived and classically transformed regenerants was a signal by PCR amplification shown with VirG primer, indicating presence of *Agrobacterium* (Table 3). Almost all

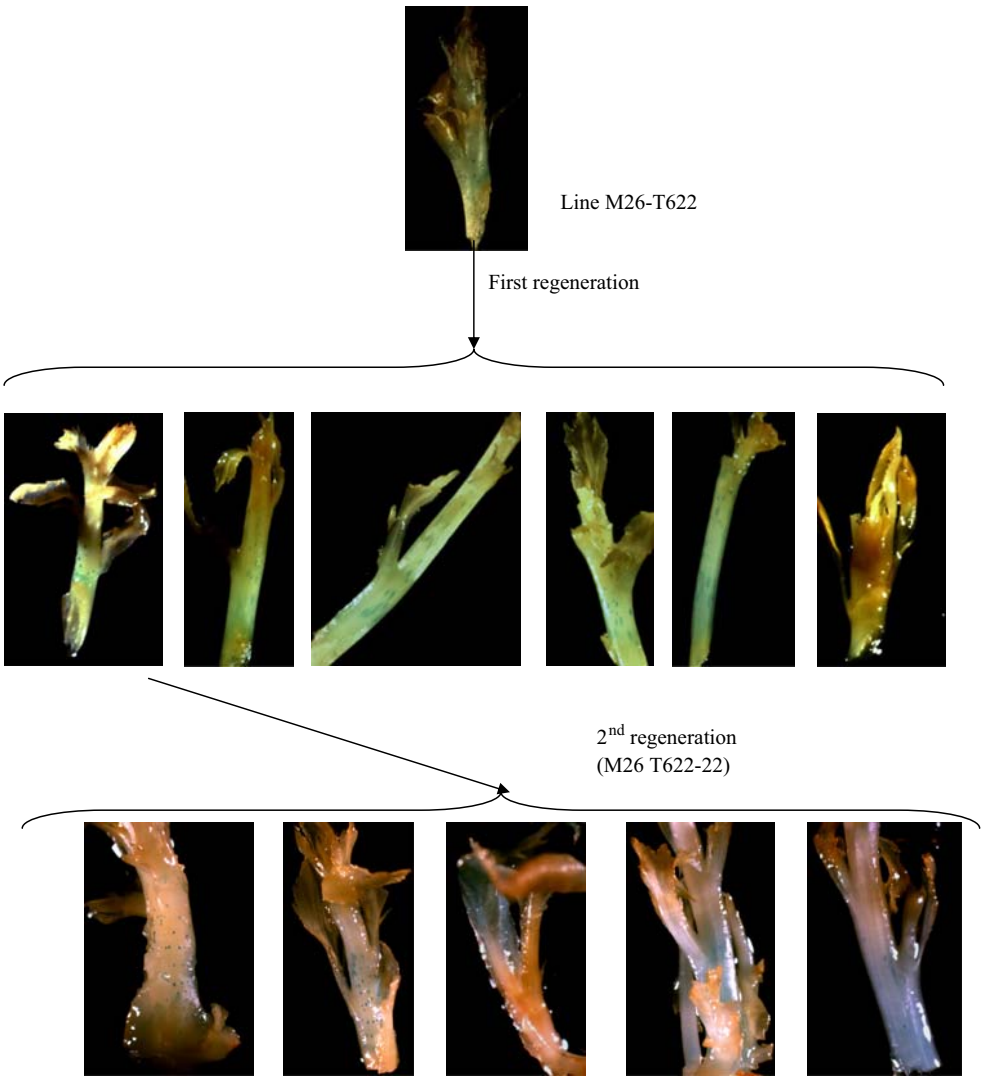

Fig. 3 Histochemical GUS assay on two successive regenerations of one pPin2ATT.35SGUSint+.nptII− markerless transformed line

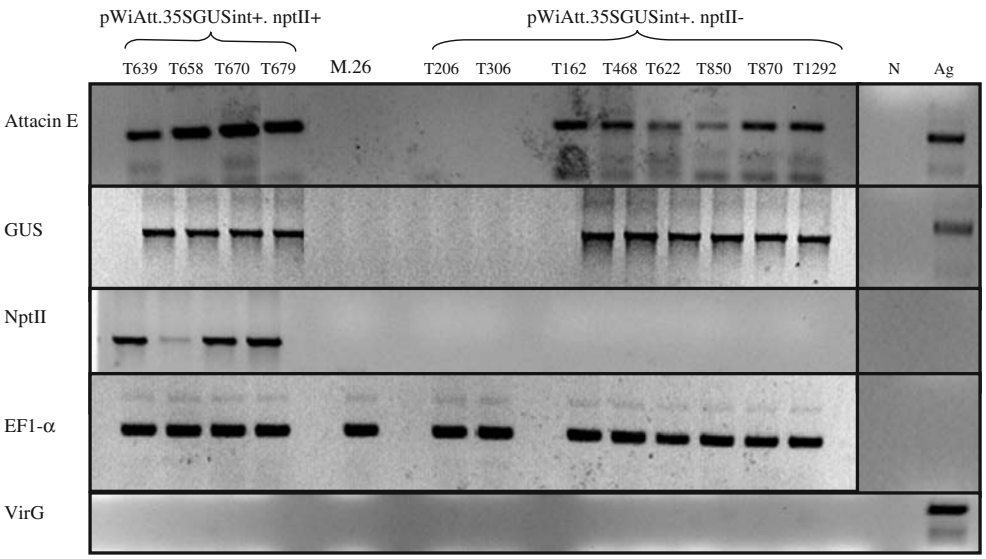

Fig. 4 Comparative RT-PCR for attacin E, GUS, nptII, EF-1α, and VirG in leaves of acclimated plants from M.26 transgenic lines obtained by markerless DNA transformation technology (T206 to T1292) and conventional transformation using nptII selection (T639 to T679) expressing the *attacinE* and *gus* genes under the control of the pin2 promoter and 35S promoter, respectively. The DNA extract from the strain of *A. tumefaciens* (*Ag*) containing the binary vector pPin2Att.35SGUSint+. nptII− was used as a positive control and the pool of all the RNA (N) from the different putative transformed lines as a negative control to check for the presence of *Agrobacterium*. Differences among transcription levels of transgenic plants were estimated after PCR (20 cycles). The EF-1α was an internal control of transcript expression. Experiments were repeated at least twice

MDTT-derived regenerants were PCR negative for VirG. Furthermore, no *A. tumefaciens* growth was observed on the proliferation medium in the different subcultures for 1 year after transformation (data not shown).

Discussion

Because selectable marker genes are integrated into the plant genome, there are concerns about widespread occurrence of these transgenes, especially antibiotic and herbicide resistance genes, in crops and in plants in the environment. Horizontal gene transfer from plants to environmental and medically significant bacteria or from plant products consumed as food to intestinal microorganisms or to human cells is generally considered to be of extremely low frequency. However, the inherent risks have not been totally addressed, and there remains both regulatory and public concern in many countries (Darbani et al. 2006). Numerous experiments have evaluated the

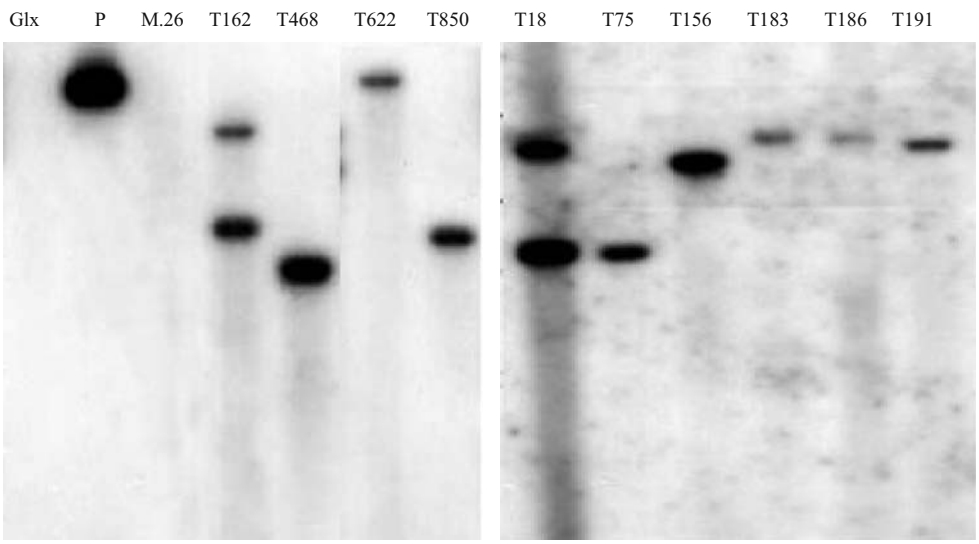

Fig. 5 Southern analysis non-transformed and putative transformant lines of Galaxy (T18, T75, T183, T186, T191) and M.26 (T162, T468, T622, T850) obtained by MDDT. Genomic DNA was digested with *Eco*RI and electrophoretically separated on 1.0% agarose gel. Southern blot was probed with the *Attacin E gene* coding region

Table 3 Efficiency of transformation of the two apple cultivars, Galaxy and M.26, with the markerless DNA transformation technology

Cultivars	Binary vector	No. of leaf pieces inoculated	No. of regenerants collected	GUS histochemical screening		PCR screening				
				No. of lines tested	% of GUS-positive lines	No. of lines tested by PCR	% of PCR-positive lines		No. of lines tested by PCR	% of PCR-positive lines
							Pin2	attacin		VirG
M26	None	75	790	250	0	150	0	0		
	pPin2tAtt.35SGUS int+.nptII−	100	1,360	566	25.6	160	nt	25	45	2
		100	1,000	300	24.2	100	nt	23.5	50	1
	pPin2MpNPR1. GUS−.nptII−	125	1,500			300	25.4	nt	60	3
		150	1,200			250	22	nt	35	1
Galaxy	none	50	530	200	0	130	0	0		
	pPin2tAtt.35SGUS int+.nptII−	131	1,300	202	14.9	110	nt	13.6	58	1
		140	900	250	13.1	100	nt	12.7	65	2
	pPin2MpNPR1. GUS−.nptII−	200	1,800			213	11.2	nt	40	0

nt not tested

possible transfer of plant DNA into microbes and mammalian cells. There are reports that bacteriophage and plasmid DNA, when fed to mice at very high levels, can later be detected in their cells (Schubbert et al. 1998), but no data exist to demonstrate that plant DNA can be transferred into and be stably maintained or expressed in mammalian cells. There are some experimental data indicating the transfer of plant DNA into bacteria under laboratory conditions but only if homologous recombination is facilitated (Kay et al. 2002). However, there is no evidence that the transgenic markers presently in use pose a health risk to humans or domestic animals. Nevertheless, some researchers and regulators have concluded that, although the transformation risk of plant-transmitted antibiotic resistance genes to pathogenic bacteria is very small, the use of markers conferring resistance to clinically relevant antibiotics should be phased out as suitable alternative technologies become available in plant biotechnology (Darbani et al. 2006). Public concerns about the issue of the environmental safety of genetically modified plants have led to a demand for technologies allowing the production of transgenic plants without selectable (especially antibiotic resistance) markers.

We describe the development of an effective transformation system for generating such marker-free transgenic plants, without the need for repeated transformation or sexual crossing. This system used the high efficiency of transformation of some apple cultivars to transform them without using selectable markers. We describe this procedure as MDTT. The system can be applied to existing transformation protocols and was tested in two apple cultivars (Galaxy and M.26) using two vectors in which the selectable marker (*nptII* for kanamycin resistance) was removed. One vector harbors the *attacin E* antimicrobial gene and the *GUS* reporter gene, thereby enabling the histochemical monitoring of transformation events.

There are basically two strategies to produce transgenic plants not containing marker genes. The simplest is the cotransformation of genes of interest and selectable marker genes followed by the segregation of the separate genes through sexual crosses. The other strategy is the use of site-specific recombinases, under the control of inducible promoters, to excise the marker genes. Recently, effective production of marker-free transgenic strawberry and apple plants was reported using a plant-adapted inducible R recombinase gene and a bifunctional, 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 nontoxic 5-fluorocytosine to cytotoxic 5-fluorouracil. With this procedure, 22% of the strawberry plants regenerated were markerless, but no data on the efficiency in apple were reported (Schaart et al. 2004). However, a downside to these procedures is that in some lines the selectable marker is not excised and is still present in the plant genome (Schaart et al. 2004; Kondrak et al. 2006). With our MDTT, we were able for the first time in tree fruit crops to regenerate marker-free transgenic plants without the need for sexual crossing, repeated transformation, or selectable markers. There is a report of a low efficiency transforma-

tion system for the production of marker-free potato plants in which the use of a selectable marker was omitted (de Vetten et al. 2003). In our case, the efficiency of transformation was 12–25% depending on the apple cultivar used. This transformation efficiency is similar to that reported for transformation of fruit using other marker-free transformation procedures, such as the multi-autotransformation procedure in pineapple sweet orange (15%; Ballester et al. 2007) and the cre-lox system in strawberry (22%; Schaart et al. 2004). However, the efficiency of transformation with this methodology is 25–30% of the efficiency of the same procedure using kanamycin as selectable marker. Usually, we were able, using kanamycin as selectable marker, to obtain up to 80% and 40% efficiency of transformation for M.26 and Galaxy, respectively.

A minority of the markerless transformants appeared to be chimeric as shown by nonuniform GUS staining of the tissues. Chimerism was also reported from the technologies for marker-free transformation in pineapple sweet orange and in citrange (Ballester et al. 2007; Domínguez et al. 2004), in lime (Domínguez et al. 2004), and in strawberry (Schaart et al. 2004). This phenomenon seems not to be specific to MDTT but is also seen in conventional transformation systems with selectable markers. This fact has been reported recently in transgenic apple, where tissue containing a mixture (chimera) of transformed and non-transformed cells was identified (Hanke et al. 2007). However, the presence of chimeric tissue in transformed plants is not necessarily an issue because the overexpression or silencing of a gene in only a proportion of cells can result in a change in the phenotype of the plants. The need is for a sufficient proportion of the cells to be transformed so that the transformed trait is stable through an indefinite number of cycles of propagation. Recent experiments using MDTT to overexpress the apple gene encoding the anthocyanin-regulating transcription factor, MYB10, in M.26 indicate that a significant proportion of (markerless) regenerant shoots appear, on the basis of red coloration, to be uniformly transformed, in addition to nontransformed shoots, and chimeric-transformed/nontransformed shoots (Aldwinckle, unpublished). It may be possible to distinguish the uniformly transformed shoots from the chimeric shoots by real-time PCR for intensity of expression of the transferred gene. Regenerants from MYB10 transformation of M.26 using *nptII* as a selectable marker also yielded chimeric-transformed/nontransformed shoots as well as uniformly transformed shoots (Aldwinckle, unpublished).

In conclusion, the MDTT reported here is the first procedure, to our knowledge, that omitted selectable markers and is efficiently accomplished in tree fruit crops. Compared to the selectable marker procedure, the MDTT has the advantage of producing selectable marker-free plants directly without any marker DNA ever being incorporated in the plant genome, but this procedure has some issues of its own. The lower efficiency of transformation is not a significant problem for the two apple genotypes in this study because the standard transformation procedure is very efficient. MDDT must be optimized for use with apple genotypes such as Golden Delicious, Pink Lady, and Pinova, whose efficiency of selectable marker transformation is reported to be low (Hanke et al. 2000; Schaart et al. 1995; Sriskandarajah and Goodwin 1998). MDTT may require additional cycles of regeneration to produce transgenic plants with uniform transgene expression, which requires additional costs and time. But, in the end, a product free of selectable markers, eliminating some of the concerns of consumers and regulatory agencies, is obtained.

In spite of our MDDT, current GE technology for transfer of gene within species still requires the uses of components based on DNA from highly divergent species. Essential components of the binary vector currently used are derived from bacterial systems, such as the T-DNA border regions and the DNA into which the gene of interest is cloned. To avoid such problem, Conner et al. (2007) have developed the concept of intragenic vectors consisting of only plant-derived DNA fragments. They have developed this type of vector for tobacco. Current sequencing of the apple genome will provide the information necessary to identify DNA fragments with functional equivalence of important vector components. Already, Conner et al. (2007) have developed some T-DNA-like regions for apple. This type of intragenic vector and MDDT will allow production of "intragenic" (Nielsen 2003), "all native" (Rommens 2004), or "cisgenic" (Schouten et al. 2006a, b) plants for highly targeted genetic improvement.

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