Plant Biotechnology Journal (2004) **2**, pp. 233–240 doi: 10.1111/j.1467-7652.2004.00067.x

Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene

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Received 6 October 2003;
revised 5 December 2003;
accepted 9 December 2003.
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Summary

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 (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 combines an inducible site-specific recombinase for the precise elimination of undesired, introduced DNA sequences with a bifunctional selectable marker gene used for the initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants. The described system can be generally applied to existing transformation protocols, and was tested in strawberry using a model vector in which site-specific recombination leads to a functional combination of a cauliflower mosaic virus 35S promoter and a GUS encoding sequence, thereby enabling the histochemical monitoring of recombination events. Fully marker-free transgenic strawberry plants were obtained following two different selection/regeneration strategies.

Keywords: marker-free transgenic plants, negative selection, site-specific recombination, strawberry.

Introduction

To date, two different approaches for the removal of selectable marker genes from transgenic plants have been described (for a recent review, see Puchta, 2003). In the first approach, the selectable marker genes and the gene of interest are introduced at different loci of the plant genome by cotransformation, after which the selectable marker gene is segregated out by crossing sexually. The second method entails the elimination of selectable markers either by transposition or by intrachromosomal site-specific recombination. The second system is the method of choice for crops that are vegetatively propagated or have a long reproductive cycle, like most fruit crops. To eliminate the selectable marker using site-specific recombination, it has to be flanked by a pair of two directly repeated specific recombination sites, allowing the exact excision of the enclosed DNA sequence in the presence of the corresponding site-specific recombinase activity. The first reported example of selectable marker elimination in plants employed the bacteriophage P1 Cre/*lox* system, comprising Cre-catalysed recombination between *lox* sites (Dale and Ow, 1991). In this study, a lox-flanked *hpt* gene was removed from transgenic plants upon re-transformation with a construct expressing the Cre-recombinase gene.

An important improvement of this early technique was reported by Zuo *et al*. (2001) using a chemically inducible artificial transcription factor for indirect transcriptional regulation of Cre-recombinase gene expression. Thus, the recombinase gene and the *lox* recombination sites could coexist without leading to premature recombination. Following the selection of transgenic tissue, chemical induction of the recombinase gene produced the desired excision events. A major drawback of this method was the formation of genetic chimeras due to incomplete DNA excision.

Sugita *et al*. (2000) described the removal of a positive selectable marker using the site-specific recombination system R/*Rs* from *Zygosaccharomyces rouxii*, in which expression of the recombinase was directly regulated by a

chemically inducible promoter. The isopentenyl transferase (*ipt*) gene from *Agrobacterium tumefaciens*, which was used as a selectable marker, leads to cytokinine overproduction and results in transgenic shoots with abnormal shooty morphology. Following the induced excision of the *ipt* gene, the appearance of normal-looking plants emerging from abnormal tissues facilitated the simple recognition of non-chimeric, marker-free plants. Although the successful use and removal of *ipt* was demonstrated in four different plant species (Ebinuma and Komamine, 2001), the system was not very efficient, and the use of the *ipt* selectable marker may require the optimization of transformation protocols due to changes in tissue culture conditions.

One way to reduce the appearance of chimeras may be to use a negative selectable marker, such as the *Escherichia coli* cytosine deaminase (*cod*A) gene, which in plant and mammalian cells has been shown to confer sensitivity to 5-fluorocytosine (5-FC) (Mullen *et al*., 1992; Stougaard, 1993). *cod*A is a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-FC to cytotoxic 5-fluorouracil (5-FU). For example, Gleave *et al*. (1999) employed the negative selection capabilities of this gene for the production of marker-free plants. They selected kanamycin-resistant transgenic tobacco plants in which lox sequences flanked the *npt*II gene together with the *cod*A gene. Transient expression of the Cre-recombinase gene allowed the selection of completely marker-free transgenic plants on 5-FC-containing medium, albeit at low frequency.

We describe the construction and application of a system consisting of a plant-adapted version of the *Z. rouxii* R recombinase and a *cod*A-*npt*II bifunctional selectable marker gene. The recombinase is ligand-regulated (post-translationally), as first described by Logie and Stewart (1995). The *npt*II component of the bifunctional marker gene is used for the selection of transgenic tissue, whereupon the *cod*A gene can be used for selection against cells that have held on to the marker despite the action of the recombinase. The application of this inducible site-specific recombination system, in conjunction with a bifunctional selectable marker, provides an effective and versatile procedure for the production of non-chimeric, marker-free transgenic plants.

Results

Construction of binary vector

A binary vector, pRCNG, was constructed that contains a unique combination of features aimed at the efficient production, selection and detection of marker-free transgenic plants (Figure 1). First of all, the R recombinase gene was completely re-synthesized to create a plant-adapted version of the gene. Next, the C-terminus of the R recombinase gene was fused to the ligand-binding domain (LBD) of the rat glucocorticoid receptor to achieve stringent post-translational regulation of recombinase activity, as originally described by Logie and Stewart (1995).

By combining the open reading frames of *cod*A and *npt*II into a hybrid enzyme, a new bifunctional selectable marker was created. The *cod*A-*npt*II hybrid gene was tested in transformation experiments with the potato variety Bintje, from which 10 independent transgenic lines were selected on kanamycin-containing shoot regeneration medium (SRM). Axillary buds of single-node cuttings taken from shoots of all 10 transgenic lines failed to grow on 5-FC-containing

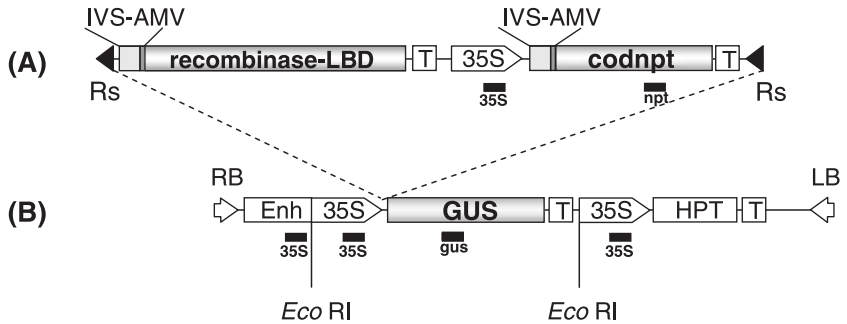

Figure 1 Diagram depicting the T-DNA of pRCNG, consisting of segment A inserted between the enhanced CaMV 35S promoter and the *gus* gene in segment B. Segment A minus one of the Rs sequences is removed on recombinase-mediated deletion. 35S-, gus- and npt-marked bars represent the probe regions used for Southern blot analysis. *Eco*RI is the restriction enzyme used for Southern blot analysis. RB, right border; LB, left border; Rs, recombination site; 35S, CaMV 35S promoter; Enh, enhancer; IVS, intron 5 of potato *gbss* gene; AMV, 5′ untranslated region of alfalfa mosaic virus; T, terminator of *Agrobacterium tumefaciens nos* gene; HPT, hygromycin resistance gene; codnpt, hybrid gene for positive (*npt*II) and negative (*cod*A) selection; GUS, β-glucuronidase reporter gene. Drawing is not to scale; internal *Eco*RI fragment is 8.7 kb and 2.9 kb before and after recombination, respectively.

propagation medium, whereas shoot growth from buds of untransformed cv. Bintje cuttings was not affected on the same medium (results not shown).

The R recombinase-LBD fusion (R-LBD) and the hybrid marker genes were located in adjacent positions and flanked by directly repeated Rs sequences. This Rs-flanked fragment was inserted between an enhanced cauliflower mosaic virus (CaMV) 35S promoter (E35S) and the β-glucuronidase (*gus*) gene, with the R-LBD gene immediately downstream and under the control of the E35S promoter (Figure 1). Dexamethasone (DEX)-induced recombinase activity would lead to excision of the fragment, leaving behind a short Rs sequence between the E35S promoter and the *gus* gene. Thus, with the *gus* gene now being positioned under the control of the E35S promoter, recombinase-mediated deletion could be easily visualized.

Strawberry transformation and selection of putative marker-free regenerants

Cultivar Calypso was used to test the pRCNG vector in strawberry because of its high shoot regeneration efficiency (Passey *et al.*, 2003). After cocultivation with AGL0(pRCNG), the leaf explants were cultured on kanamycin-containing selective SRM for 1 month. Then, two strategies were followed for the selection of marker-free transgenic strawberry plants. In the early negative selection approach, half of the leaf explants were incubated overnight in liquid medium supplemented with 10 μM DEX to induce recombinase activity, and the leaf explants were subsequently transferred to kanamycin-free SRM containing 1 μM DEX for sustained R recombinase induction and 150 mg/L 5-FC for selection against cells retaining the marker.

Following the early approach, 126 regenerating shoots and shoot-like structures were isolated from the leaf explants, and fragments from all regenerants were subjected to a GUS-staining assay. Partially or completely blue shoot fragments resulting from recombinase-mediated removal of the sequences between the 35S promoter and the *gus* gene were observed for 28 of these shoots. Most of the regenerants were in the form of small shooty clusters and were often vitrified. Attempts to generate shoots from those on shoot propagation medium without selection eventually gave rise to four normal-growing shoots, 20 weeks after the start of the experiment. These putative marker-free shoots were denoted as 'early' shoots.

In the alternative, delayed (negative) selection strategy, the remaining half of the leaf explants were transferred to fresh kanamycin-containing SRM, from which, eventually, 56 individual kanamycin-resistant shoots were regenerated and propagated on shoot propagation medium containing 50 mg/L kanamycin. With 51 shoots from these lines, the marker elimination procedure was started by treating leaf explants with 10 μM DEX, as described above, to induce recombinase activity. The leaf explants were subsequently subjected to a second round of shoot regeneration in the presence of 1 μM DEX and 150 mg/L 5-FC, and secondary regenerants were obtained from all lines 35 weeks after the start of the experiment. These putative marker-free regenerants ('late' shoots) were propagated and subjected to further analysis.

A remarkable observation was made when leaf explants of the kanamycin-resistant plants (primary regenerants), destined for the delayed selection approach, were subjected to a histochemical GUS assay immediately following DEX treatment. As a control, untreated leaf explants from the same plants were also assayed for GUS activity. In 32 of 51 independent transgenic plants treated with DEX and tested for GUS activity, staining was observed, the remainder being GUS-negative. This suggests that DEX induced recombination in leaf explants of at least 62% of the primary regenerants. Surprisingly, for all lines tested, a similar GUS-staining intensity and pattern was observed for untreated leaf explants of corresponding plants, indicating that (partial) recombination had already occurred before the start of DEX treatment. The primary regenerants, which may have undergone premature recombination, were not subjected to further molecular analysis.

Analysis of putative marker-free plants

The four putative marker-free plants from the early selection and 13 randomly selected putative marker-free plants obtained via the delayed selection protocol were tested for GUS activity. Two of the four early selected putative marker-free plants (E2 and E4) showed strong GUS staining, whereas, in a third plant (E1), a very faint staining was observed and, in the fourth (E3), no GUS staining was observed (data not shown). Of the 13 supposedly marker-free plants obtained by delayed selection, 10 showed strong GUS staining and, in three, reduced staining was found. All four putative marker-free plants from the early selection and four of the strongly GUS-positive putative marker-free plants from the late selection (L1–4) were subjected to further molecular analysis.

Polymerase chain reaction (PCR) and Southern blot analysis of genomic DNA showed that only line E1 still contained a copy of the marker gene (Figure 2A,B, *npt*II panels). Hybridization

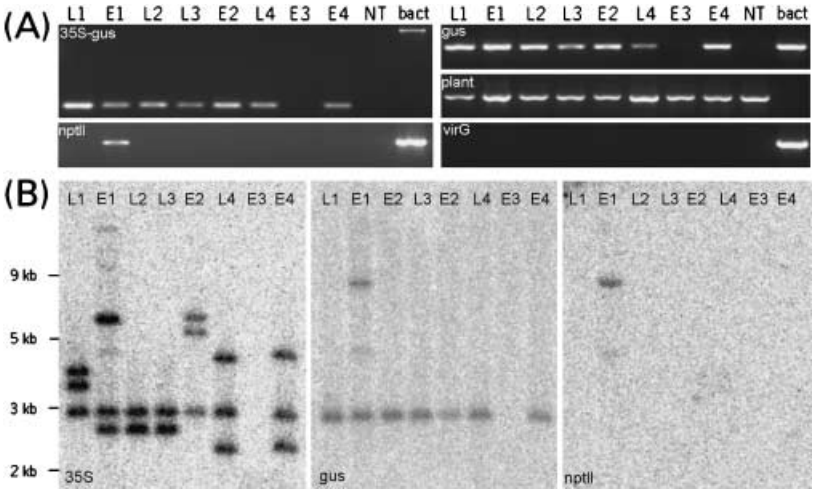

Figure 2 Molecular analysis of putative marker-free plants. E1–E4, putative marker-free transgenic plants obtained through early negative selection; L1–L4, putative marker-free transgenic plants obtained through late negative selection; NT, non-transgenic control; bact, *Agrobacterium tumefaciens* AGL0(pRCNG) as non-recombined positive control. (A) Polymerase chain reaction analysis using primers specific for 35S-promoter and *gus* gene, *npt*II gene, *gus* gene, endogenous strawberry polygalacturonase gene (plant) and *A. tumefaciens vir*G gene. (B) Southern blot analysis of *Eco*RI-digested DNA, hybridized with 35S, *gus* and *npt*II probes.

of DNA from line E1 with the *gus* probe revealed three *Eco*RI fragments (Figure 2B, *gus* panel), one weakly and two strongly hybridizing fragments, one of which also hybridized to the *npt*II probe. Although the size of this common hybrid fragment corresponds to the size of the full-length *Eco*RI fragment that was part of the original T-DNA, the lack of expected hybridization with the 35S probe precludes such a straightforward explanation (Figure 2B, 35S panel). PCR analysis demonstrated that one of the four plants obtained from the early selection (E3) was likely to be a non-transgenic escape, as it lacked not only the marker and the 35S promoter, but also the *gus* gene, as confirmed by Southern blot analysis using a *gus* probe.

Using a primer pair consisting of a forward primer specific for the CaMV 35S promoter and a reverse primer specific for the *gus* gene, PCR analysis revealed the small fragment of expected size in all GUS-positive plants, including that which still contained the marker gene. The appearance of this fragment indicated that a 6.1 kb deletion took place that positioned the *gus* gene immediately downstream of the enhanced CaMV 35S promoter. This indicated that complete recombinase-mediated removal of the recombinase and the selectable marker occurred in all GUS-positive plants. In line E1, however, amplification with these primers might also have yielded a much longer PCR fragment, amplified from non-recombined T-DNA as shown for the pRCNG template (Figure 2A, 35S-gus primers, bacterial control), if the 9.3 kb fragment observed in the Southern blot hybridizing with the 35S probe had represented a complete T-DNA. The absence of this larger fragment encompassing part of the 35S promoter, the recombinase-LBD, the complete selectable marker and part of the *gus* gene might be due to the PCR conditions being biased towards the amplification of small fragments. However, it is also likely that the 9.3 kb fragment of E1 is not derived from a full-length T-DNA, but instead corresponds to a truncated T-DNA copy.

Southern blot analysis using the 35S probe yields information on the number of T-DNA copies integrated into the strawberry genome. Four of six marker-free transgenic plants displayed two *Eco*RI fragments in addition to the internal one present in all GUS-positive lines, which would seem to indicate the integration of one T-DNA. Apparently, the other two plants (L2 and L3) only showed one additional *Eco*RI fragment, but the relatively high intensity of this additional fragment, as compared with the internal one, indicated the presence of different fragments of similar length.

Discussion

We have developed an effective method for the production of transgenic plants from which selectable marker genes have been removed. The unique combination of a chemically inducible recombinase activity and a bifunctional selection system allowed the straightforward production of completely marker-free transgenic plants, without the need for repeated transformation or sexual crossing.

The described method was tested by applying two different selection procedures. The efficiencies of the two negative selection procedures were found to differ considerably. The delayed selection procedure, starting with leaf explants from fully grown kanamycin-resistant transformants, produced more than 30 putative marker-free plants. Molecular analysis revealed that all four selected plants were completely free of selectable markers. On the contrary, early selection of marker-free plants from leaf explants that had only just begun to yield shoots (after 4 weeks of positive selection)

produced only very few marker-free transgenic plants. Here, one of the putative marker-free plants appeared to be non-transgenic, indicating that positive selection was insufficient. In another plant, the selectable marker was still present. Southern blot analysis suggested that it was likely that, in this case, more than one T-DNA was integrated into the genome, and that one truncated, incomplete T-DNA copy had resulted in the loss of recombination capability and *cod*A expression. For the two remaining putative marker-free plants from the early selection, the selection markers were proven to be eliminated completely. As the number of leaf explants originally infected with pRCNG was roughly the same for the two selection procedures, delayed selection was much more effective. For early negative selection, however, increased efficiencies may be obtained by optimizing the timing of selection procedures.

One of the key features of the system presented here is the hybrid combination of a positive and a negative selectable marker gene. For positive selection of transgenic tissue, the *npt*II gene, conferring kanamycin resistance, was used, because of its effectiveness in strawberry transformation experiments (Schaart *et al*., 2002). The *E. coli cod*A gene has been successfully used as a negative selectable marker in plant transformations, especially at the stage of shoot regeneration (Schlaman and Hooykaas, 1997), and has also been used as a selection marker in another marker removal scheme (Gleave *et al*., 1999). Concentrations ranging from 50 to 500 mg/L were described to be effective for negative selection at the regeneration stage (Schlaman and Hooykaas, 1997). In our hands, 5-FC at a concentration of 150 mg/L was sufficient for the regeneration of transgenic plants, which were completely devoid of marker genes.

The use of a chemically inducible recombinase activity allowed the simultaneous introduction of the R recombinase gene and the Rs recombination sites, so that there was no need for re-transformations or sexual crossing in order to combine the separated recombinase and its recombination sites after transgenic plants had been selected. Although marker-free plants could be obtained in an effective way using the inducible R recombinase activity, it was demonstrated by GUS staining that significant recombination had already occurred in non-induced leaves of kanamycin-resistant transgenic shoots from the delayed selection. As these shoots showed sustained growth on kanamycin-containing medium, and as they could only be GUS-positive due to deletion of the DNA fragment carrying the selectable marker, this implied that the plants were chimeric for the selectable marker. Apparently, early partial elimination of the selectable marker did not affect the initial positive selection ability necessary for the production of the transgenic plants.

The observation that significant recombination had already taken place before treatment with DEX suggested that the control exerted by LBD was incomplete. Up to now, the principle of the LBD-mediated regulation of recombinase activity has not been clarified; it may be based on the prevention of nuclear localization of the recombinase protein, on the inability of complexed recombinase to engage in recombination, or both (Picard and Yamamoto, 1987). In contrast with the direct enzyme activity control system (post-translational control) employed in this study, the steroid receptor LBD has also been used for the indirect control of enzyme activity (transcriptional control) by fusing it to the transcription factor that regulates the expression of the target gene. For example, Aoyama and Chua (1997) described the use of a hybrid transcription factor fused with the LBD of the rat glucocorticoid receptor, and demonstrated that this allowed stringent control of the luciferase reporter gene. The same system used for *gus* reporter gene regulation also allowed tight regulation in most transgenic lines studied (Ouwerkerk *et al*., 2001). Kunkel *et al*. (1999), however, showed leakiness of this LBD-mediated inducible system when they used it for the regulation of *ipt* expression. Possibly, non-induced gene expression levels are too low to be detected by the reporter genes used by Aoyama and Chua (1997) and Ouwerkerk *et al*. (2001). It is only when even low expression levels of a reporter lead to phenotype differences, as with the *ipt* gene, that background activity of the system becomes evident. Our system is likely to be sensitive to low expression levels too, as it is essentially an irreversible system, resulting in the accumulation of recombination events in time and a concomitant increase in GUS activity levels. Although such a low basal expression level is generally seen as undesirable, in our system it can be beneficial, as the elimination of undesired sequences continues as long as the recombinase gene is present, even under non-inductive situations. On the other hand, premature recombination might reduce the frequency of transgenic plants with single-copy T-DNA insertions, as untimely removal of the single marker would render them kanamycin sensitive at an early stage. However, this does not seem to be the case, in view of the relatively high number of single-copy transformants obtained in this study.

So far, other methods describing the production of marker-free plants without the requirement of sexual crossing or repeated transformations have not been very efficient or the application has been limited to specific species and varieties. For example, de Vetten *et al*. (2003) reported on a transformation system for the production of marker-free potato

plants in which the use of a selectable marker was omitted. The practicality of their approach of using PCR to separate transgenic from untransformed shoots relies on high transformation efficiencies. Zuo *et al*. (2002) reflected on the use of regeneration-promoting genes, such as the *ipt* gene, for the production of marker-free plants, but application to new systems generally requires time-consuming re-optimization of existing transformation protocols. The strategy for the production of marker-free plants described by us is effective and is, in principle, widely applicable to any plant species for which transformation protocols have been developed. Our system differs from other marker-free transformation approaches in the possibility of selection for completely marker-free transgenic plants, so that the formation of genetic chimeras, a major drawback of other systems, is prevented. In order to substantiate the wide applicability of the system described here, we applied it to the apple cultivar Elstar, which can only be transformed at low frequencies (Puite and Schaart, 1996), and were successful in obtaining completely marker-free transgenic apple shoots (results not shown). In addition, in tobacco, the use of chemically induced recombination activity, combined with secondary regeneration under negative selection conditions, was quite effective in obtaining completely marker-free transgenic plants (results not shown).

Experimental procedures

Construction of binary plasmid

The binary plasmid pRCNG (Figure 1) is based on pMOG22 (Goddijn *et al*., 1993). The R recombinase coding sequence was re-synthesized, increasing its G + C content from 41% to 49% and its frequency of favourable XXG/C codons from 41% to 63%, without altering the amino acid composition. For this, 24 sense and antisense oligonucleotides with an average size of 81 nucleotides were used in an overlap extension PCR method, as described by Ho *et al*. (1989) and Rouwendal *et al*. (1997); this yielded the full-length product. The LBD of the rat glucocorticoid receptor was translationally fused to the C-terminus of the R recombinase gene. The LBD sequence was obtained from the rat cDNA clone 6RGR (kindly provided by K. R. Yamamoto) by overlap extension mutagenesis that eliminated an internal *Eco*RI site (Miesfield *et al*., 1986). The coding sequence of the hybrid selectable marker gene consists of the *cod*A gene, isolated from *E. coli* strain JM109 by PCR using primers 5′-GTGAACCATGGCTAATAACGCTTTACAAACAA-3′ and 5′-GCAGTGGATCCACGTTTGTAATCGATGG-3′, translationally fused to the *npt*II gene, isolated from pBIN19 using primers 5′-TCGCAGATCTGAACAAGATGGATTGCACG-3′ and 5′-GCTCAGGATCCCGCTCAGAAGAACTCGTC-3′. Both hybrid recombinase R-LBD and *cod*A-*npt*II coding sequences are flanked by a downstream nopaline synthase gene terminator (t*nos*) and an upstream translational enhancer consisting of the alfalfa mosaic virus (AMV) 5′ untranslated region followed by intron 5 from the potato *gbbs* gene (IVS) (Jobling and Gehrke, 1987; van der Leij *et al*., 1991). The *cod*A-*npt*II hybrid gene was regulated by the CaMV 35S promoter. The combination of the promoterless recombinase R-LBD gene and the *cod*A-*npt*II hybrid gene is flanked by 58 bp of directly repeated recombination sites (*Rs*) which were isolated from *Z. rouxii* total DNA by PCR using primers 5′-AGGCGAGATCTTATCACTGT-3′ and 5′-GTCACGGATCCACGATTTGATGAAAGAAT-3′. This Rs-flanked segment separates an enhanced CaMV 35S promoter and a *gus* reporter gene in such a way that its recombinase-mediated elimination will lead to GUS activity. Initially, this CaMV 35S promoter controls the recombinase R-LBD hybrid gene. The plasmid pRCNG also contains an *hpt* gene available for control experiments.

Transformation of strawberry and elimination of selectable marker genes

For plant transformation, the binary construct pRCNG was transferred to *A. tumefaciens* strain AGL0 (Lazo *et al*., 1991). Transformation of the strawberry cultivar Calypso was essentially performed as described by Schaart *et al*. (2002). Leaf explants were infected with AGL0(pRCNG) and cocultivated for 3 days on SRM consisting of Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) glucose, 5 μM thidiazuron, 1 μM 1-naphthaleneacetic acid (NAA) and 0.4% (w/v) gelrite, after which the leaf explants were transferred to selective SRM containing 250 mg/L cefotaxime for the elimination of *A. tumefaciens* and 150 mg/L kanamycin for the selection of transgenic tissue. After 1 month on this medium, leaf explants were subcultured in two different ways. In the first case, the leaf explants were incubated overnight in liquid MS medium supplemented with 10 μM DEX for the activation of the R recombinase protein, after which they were transferred to fresh SRM containing 250 mg/L cefotaxime, 1 μM DEX and 150 mg/L 5-FC each month. In this medium, 5-FC is present for the negative selection of marker (*cod*A)-free regenerating shoots. Alternatively, after the first month on selective SRM, the leaf explants were transferred to fresh SRM containing 250 mg/L cefotaxime and 100 mg/L kanamycin in order to regenerate kanamycin-resistant shoots first. These shoots were isolated from the leaf

explants and subcultured on shoot propagation medium (MS medium with 3% (w/v) sucrose and 0.9% (w/v) Daishin agar) supplemented with kanamycin at a concentration of 25 mg/L. Shoots that rooted on this medium were regarded as kanamycin-resistant transgenic shoots. Leaf explants from these shoots were incubated overnight in liquid MS medium supplemented with 10 μM DEX and then transferred to SRM containing 1 μM DEX and 150 mg/L 5-FC to obtain regenerants again. Putative marker-free plants obtained following both approaches were rooted and propagated on shoot propagation medium containing 150 mg/L 5-FC and were moved to the greenhouse for further analysis.

Histochemical GUS assay

Histochemical GUS staining of leaves was carried out as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40.

PCR and DNA gel-blot hybridization analysis

DNA was isolated from young folded leaves from greenhouse-grown plants according to the method described by Doyle and Doyle (1987), but including 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. In order to obtain representative sampling for DNA isolation, leaf material was pooled from several individuals from the same transgenic line. For PCR analysis of the putative marker-free plants, the following primer sets were used (see also Figure 1). B4 primers (5′-CTCTTCCTCTCTGTAACACC-3′ and 5′-CTGCTGCATCTCTTCCTCTACCAT-3′), corresponding to a strawberry polygalacturonase-like gene (GenBank accession no. AY280662), were used as a quality check of the template DNA. Vir primers (5′-GCCGGGGCGAGACCATAGG-3′ and 5′-CGCACGCGCAAGGCAACC-3′), which amplify the *A. tumefaciens vir*G gene, served to exclude the presence of *A. tumefaciens* bacteria. Gus primers (5′-CTGTAGAAACCCCAACCCGTG-3′ and 5′-CATTACGCTGCGATGGATCCC-3′) were used for the detection of the T-DNA. Primers CodNpt-up and -dw (5′-AAGGTGATTGCCAGCACACA-3′ and 5′-TACGTGCTCGCTCGATGCGA-3′, respectively) allowed the detection of selectable marker sequences. 35S and gus primers (5′-CCACTATCCTTCGCAGACC-3′ and 5′-TATCTGCATCGGCGAACTGA-3′, respectively) were used for the detection of recombination events. As control templates, DNA from non-transgenic cv. Calypso plants and *A. tumefaciens* AGL0(pRCNG) bacteria were included in each PCR test. For DNA gel-blot hybridization analysis, 5 μg of DNA of control and transgenic plants was digested with *Eco*RI and separated by electrophoresis on a 0.8% (w/v) agarose gel. The DNA was then transferred to a Hybond-N membrane (Amersham) and hybridized with the ^{32}P-labelled coding region of *npt*II, *gus* and a PCR fragment of the CaMV 35S promoter sequence, respectively, as described earlier by Puite and Schaart (1996).

Acknowledgements

We thank Nicky Hallsted for her kind help with the molecular analysis of the transgenic plants.

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