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ORIGINAL PAPER

The pollen- and embryo-specific *Arabidopsis DLL* promoter bears good potential for application in marker-free Cre/*lox*P self-excision strategy

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Abstract
Key message **Marker-free transgenic plants can be generated with high efficiency by using the Cre/*lox*P self-excision system controlled by the pollen- and embryo-specific *Arabidopsis DLL* promoter.**
Abstract In this work, we aimed to study the feasibility of using the pollen- and embryo-specific *DLL* promoter of the At4g16160 gene from *Arabidopsis thaliana* in a Cre/*lox*P self-excision strategy. A Cre/*lox*P self-excision cassette controlled by the *DLL* promoter was introduced into the tobacco genome via *Agrobacterium*-mediated transformation. No evidence for premature activation of the Cre/*lox*P system was observed in primary transformants. The efficiency of *npt*II removal during pollen and embryo development was investigated in transgenic T_1 progenies derived from eight self- and four cross-pollinated T_0 lines, respectively. Segregation and rooting assays were performed to select recombined T_1 plants. Molecular analyses of these plants confirmed the excision event in all analysed T_0 lines and marker-free transgenic T_1 plants were obtained with efficiency of up to 96.2 %. The *Arabidopsis DLL* promoter appears to be a strong candidate to drive Cre-mediated recombination not only in tobacco as a model plant, but also in other plant species.

Keywords *Agrobacterium tumefaciens* · Cre/*lox*P · Marker-free transgenic plants · Pollen- and embryo-specific promoter · Self-excision · Tobacco

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Introduction

Selectable marker genes enable selection of the small number of cells that are able to include foreign DNA. The presence of such genes in the genome of transgenic plants, especially those encoding for resistance to antibiotics or herbicides, has caused considerable public concern about their potential adverse impact on human health and environment (Costa-Font et al. 2008; Nicolia et al. 2014).

Several approaches to eliminate selectable markers have been described including co-transformation, transposon-based transgene excision, homologous recombination or site-specific recombination (reviewed by Scutt et al. 2002; Gidoni et al. 2008; Woo et al. 2011 or Tuteja et al. 2012). Over the recent years studies have focused on the site-specific Cre/*lox*P recombination system. The first commercial marker-free transgenic maize (LY038) has been developed (Ow 2007).

The Cre/*lox*P recombination system consists of the gene for *cre* recombinase and of two *lox*P sites. Cre recombinase mediates a recombination event ending with excising the

DNA sequence placed between two directly oriented *lox*P sites (Gilbertson 2003). Individual approaches exploiting the Cre/*lox*P system differ in duration of *cre* expression, which can be constitutive, transient or temporal. The constitutively expressed *cre* gene is delivered to targeted *lox*P sites by cross-pollination (Odell et al. 1990; Hoa et al. 2002; Chakraborti et al. 2008) or re-transformation (Dale and Ow 1991; Russel et al. 1992). To reduce undesired long-term effect of the *CRE* protein (Coppoolse et al. 2003), approaches based on transient and temporal *cre* expression were developed. Transient *cre* expression has been achieved by application of purified Cre protein (Will et al. 2002) and virus- or *Agrobacterium*-mediated *cre* expression (Kopertekh and Schiemann 2005; Kopertekh et al. 2012).

In the temporal expression approach, the *cre* recombinase and the selectable marker genes are placed between two *lox*P sites as a part of the same T-DNA. Upon activation, the Cre recombinase removes the marker gene as well as its own sequence. The excision event can be controlled by using inducible promoters activated by heat shock (Zhang et al. 2003; Liu et al. 2005; Wang et al. 2005; Cuellar et al. 2006; Roy et al. 2008), chemically (Zuo et al. 2001; Sreekala et al. 2005; Zhang et al. 2006, 2009; Petri et al. 2012; García-Almodóvar et al. 2014), by salicylic acid (Ma et al. 2008, 2009); or by using promoters of genes with a high degree of specific expression such as microspore *NTM19* (Mlynarova et al. 2006), pollen/seed-specific *PAB5* (Luo et al. 2007), embryo-specific *APP1* (Li et al. 2007), germline-specific *SDS* and *AP1* (Verweire et al. 2007), flower-specific OsMADS45 (Bai et al. 2008), seed-specific *CRUC* (Moravcikova et al. 2008; Boszorádová et al. 2014), seed-specific napin (Kopertekh et al. 2009) or embryo-globulin *REG-2* (Chong-Pérez et al. 2013).

However, efficiency of excision varies greatly. For example, when *cre* expression was regulated by the embryo-specific *CRUC* promoter, an excision efficiency of 10.2 % was achieved (Moravcikova et al. 2008). Unexpected complications with premature excision of the marker gene were observed suggesting that a more reliable promoter is needed. TAIR database searches identified the promoter *DLL* of *Arabidopsis* At4g16160 gene as highly tissue-specific and robust towards premature activation. The activity of *DLL* in *Arabidopsis* is specifically limited to developing pollen grains (Honys and Twell 2004), young ovules and developing embryos (Drea et al. 2006). Experiments performed on transgenic tobacco using the *gus* gene-reporter system (Jopcik et al. 2014) showed activity of *DLL* in pollen grains and germinating tubes as well from the middle torpedo stage of the developing embryo. No GUS activity was detected in leaves and stems. In addition, among the five pollen- and/or embryo-specific promoters tested, only the *DLL* promoter showed activity consistent with the predicted pattern (Jopcik et al. 2014).

Therefore, in this work, we studied the feasibility of using the promoter *DLL* from *Arabidopsis* to drive Cre-mediated excision of the selectable *npt*II gene in tobacco. The efficiency of *npt*II removal in pollen and embryo was investigated in transgenic T_1 progenies of selected self- and cross-pollinated T_0 plants.

Materials and methods

Vector construction

The *DLL* promoter sequence was previously isolated as a 1644 bp PCR fragment of the At4g16160 gene from *Arabidopsis thaliana* (Jopcik et al. 2013).

The pZP6 construct (Fig. 1a) was prepared by cloning *dCaMV35S/gus/nosT/lox*P*/DLL/cre*^{INT}*/nosT* and *nosP/nptII/nosT/loxP* fragments into the low copy number binary vector pUN (Vaculkova et al. 2007) as described by Poloniová et al. (2012).

The plasmid pZP6 was evaluated for stability and recombination events in *Escherichia coli* DH5αF′ according to the protocol by Mlynarova and Nap (2003).

The binary vector pZP6 was introduced into *Agrobacterium tumefaciens* strain AGL0 and its stability was verified by restriction analyses after re-transformation into *E. coli*.

Plant material and transformation

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed using the leaf disc transformation protocol described by Mlynarova et al. (1994). The transformed tobacco tissues were selected on medium with 50 mg l^{-1} kanamycin. Regenerated shoots were rooted in the presence of 50 mg l^{-1} kanamycin. Selected transgenic plants were transferred to the soil, cultivated in greenhouse conditions and used to conduct self- and cross-pollination with the wild type.

β-Glucuronidase assays

Histochemical GUS assays were conducted as described by Jefferson et al. (1987). Leaf explants/seedlings were incubated in 1 mmol l^{-1} 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Duchefa, Netherlands), 50 mmol l^{-1} phosphate buffer (pH 7) at 37 °C in the dark overnight. To improve colour contrast, the tissues were washed in 70 % (v/v) ethanol.

Fluorimetric GUS assays were performed as described by Mlynarova et al. (1994). GUS activity was expressed in

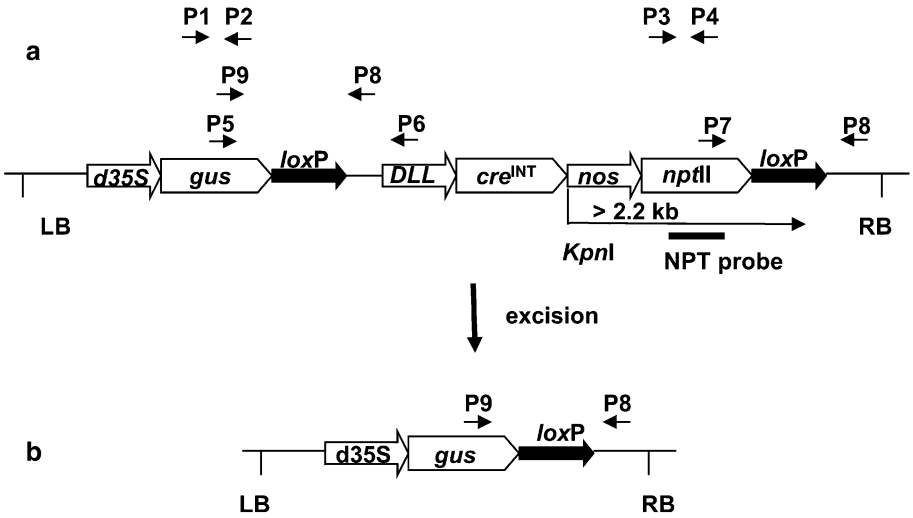

Fig. 1 The plant binary vector pZP6 used in the transformation experiments. **a** T-DNA configuration consisting of the β-glucuronidase (*gus*) gene driven by the double *dCaMV 35S* promoter (*d35S*); the intron-containing *cre* recombinase gene (cre^{INT}) under control of *DLL* promoter and the neomycin phosphotransferase (*nptII*) gene driven by the *nos* promoter. All three genes were terminated by the *nosT*. *Black arrows* indicate the presence and orientation of the *loxP* sites. The restriction site used for Southern blot analyses is indicated as well as predicted fragment size. The positions of primers used for PCR analyses are indicated as P1/P2, P3/P4, P5/P6, P9/P8 and P7/P8. **b** The T-DNA configuration generated after excision of the floxed DNA. The positions of primers used for PCR analyses are indicated as P9/P8

picomoles of methylumbelliferone released per min per μg of soluble protein. The concentration of proteins was determined according to Bradford (1976).

Segregation assays

Two segregation assays were used to test for kanamycin resistance in progenies of the transgenic plants. In the germination assay, surface-sterilised seeds (about 100 seeds per plant) germinated on MS medium (Murashige and Skoog 1962) containing 1 % (w/v) sucrose, 0.8 % (w/v) agar and 50 mg l^{-1} kanamycin. Three weeks later, the seedlings were evaluated for kanamycin resistance. Green seedlings were considered to be kanamycin-resistant (Km^R) and yellow or pale seedlings as kanamycin-sensitive (Km^S).

A rooting assay was carried out according to Moravcikova et al. (2008). Surface-sterilised tobacco seeds (about 100 seeds per plant) were germinated on MS medium (Murashige and Skoog 1962) containing 1 % (w/v) sucrose and 0.8 % (w/v) agar under non-selective conditions. After 2 weeks the seedlings were transferred into 100-ml glass culture vessels onto fresh MS medium and grown for 6 weeks. Next, roots were removed from plants and each plant was multiplied vegetatively into two clones. The first clone was allowed to re-root in the presence of 50 mg l^{-1} kanamycin. The plants that formed roots in 3 weeks were evaluated as kanamycin-resistant (R+), whereas non-rooted plantlets were considered kanamycin-sensitive (R−). The second clone was rooted without the presence of antibiotics. These plants were used for DNA analyses.

PCR analyses

Genomic DNA from the leaf tissues of tobacco plants was isolated using the protocol of Chen et al. (1992). The primers used in PCR analyses, the size of expected amplicons and other relevant descriptions are listed in Table 1. The respective positions of the primers are given in Fig. 1. The PCR reaction mixture of 25 μl contained 100–200 ng of DNA template, 20 pmol of each primer, 0.2 mmol l^{-1} dNTPs, 1× PCR buffer, 2.5 mmol l^{-1} $MgCl_2$ and 1 U FIREPol Taq DNA polymerase (Solid BioDyne, Estonia). Step one of the PCR was performed at 95 °C for 4 min, followed by 35 cycles of 95 °C for 45 s, 62 or 64 °C (see Table 1) for 45 s and 72 °C for 90 s. The last step was performed at 72 °C for 10 min.

Some of the PCR amplicons were isolated using the QIAEX® II Gel Extraction Kit (Qiagen, Germany), cloned into the pGEM®-easy vector and commercially sequenced. Alignment of the obtained sequences was performed using the CLUSTALW2 program (Thompson et al. 1994).

Southern blot analyses

Genomic DNA (10 μg) was digested with restriction enzyme KpnI, separated on a 1 % (w/v) agarose gel and

Table 1 Primers used for PCR analyses, annealing temperature in PCR reaction and expected size of PCR products

Primer code	Primer	Sequence	Annealing (°C)	Expected size (bp)	Purpose
P1	Forward	5′-GTT CCT GAT TAA CCA CAA ACC-3′	62	744	*gus* gene internal fragment
P2	Reverse	5′-TGC ACA CTG ATA CTC TTC A-3′			
P3	Forward	5′-GAT GGA TTG CAC GCA GGT TCT-3′	64	552	*npt*II gene internal fragment NPT probe
P4	Reverse	5′-ATG GGT CAC GAC GAG ATC ATC-3′			
P5	Forward	5′-GCA GCA GGG AGG CAA ACA ATG AAT-3′	62	589	*gus-loxP-DLL* fragment
P6	Reverse	5′-GTC CCG AAA GGA ATT GAA GTT GAT GG-3′			
P7	Forward	5′-GGT GCC GAT ATC ATT ACG-3′	62	781	*nptII-loxP-RB end* fragment
P8	Reverse	5′-AAG AAT TCG AGC TCG GTA CC-3′			
P9	Forward	5′-TCA GTG TGC ATG GCT GGA TA-3′	62	653	*gus-loxP-RB end* fragment (excision of the floxed DNA) *gus-loxP* fragment (non-excised)
P8	Reverse	5′-AAG AAT TCG AGC TCG GTA CC-3′		716	

blotted onto a positively charged nylon membrane (Roche, Switzerland). An NPT-specific probe was prepared using PCR with the primer set P3/P4 and non-radioactively labelled using the DIG Probe Synthesis Kit (Roche, Switzerland). Hybridisation was performed in DIG easy hyb hybridisation solution (Roche, Switzerland) at 42 °C according to the manufacturer's instructions. Hybridisation signals were visualised by DIG Nucleic Acid Detection Kit (Roche, Switzerland).

Results

The pollen- and embryo-specific *Arabidopsis DLL* promoter was used to drive the *cre* recombinase. The T-DNA of the corresponding plant transformation vector pZP6 is given in Fig. 1a. Figure 1b shows the final outcome of Cre-mediated excision in transgenic tobacco plants.

Generation and proof of T_0 plants

The pZP6 T-DNA was introduced into the tobacco genome via *Agrobacterium tumefaciens*. Putative transgenic shoots were initially screened for kanamycin resistance and for GUS activity. Transformation efficiency (56.6 %) was normal; therefore, we assumed that no premature activity of the *DLL* promoter occurred during regeneration of transformed cells.

A set of histochemically GUS-positive T_0 plants were subjected to PCR analyses with primers P1/P2, P3/P4, P5/P6 and P7/P8. PCR products corresponding to the predicted sizes of 744 bp (P1–P2), 552 bp (P3–P4), 589 bp (P5–P6) and 781 bp (P7–P8) were detected in all analysed T_0 plants. Examples of the PCR analyses with the primer sets P5/P6 and P7/P8 are given in Fig. 2a and b respectively.

Further, PCR analyses were used to detect possible ectopic activation of *DLL* and thus premature excision of the *npt*II gene. Unfortunately, there were limited alternatives for designing the proper primer set (Fig. 3a). By using primers P9/P8, two PCR products could be generated, the first of 716 bp (P9–P8) derived from non-recombined T-DNA templates and the second of 653 bp (P9–P8) from the recombined T-DNA templates. In all analysed T_0 plants, only a 716 bp P9–P8 PCR product was detected (Fig. 2c) pointing to lack of premature excision of the *npt*II gene in the T_0 plants. The P9–P8 amplicon (line T_0-2) was isolated and sequenced. Pairwise alignments showed the identity of the amplicon with the corresponding non-recombined sequence of the pZP6 T-DNA (Fig. 3b).

The T_0 plants were screened by Southern blot hybridisation using the NPT probe after digestion of DNA with KpnI to determine the number of the right border fragments. Based on the restriction map of pZP6 (Fig. 1a), the probe was expected to hybridise with fragments larger than a 2.2-kb border fragment. The number of detected fragments corresponded to the number of integrated transgenes. The number of *npt*II gene copies varied from 1 to 5 (Fig. 4).

Eight of the analysed T_0 plants with single- or two-copy integrations were identified, transferred to in vivo conditions and subjected to self- and cross-pollinations.

Efficiency of *npt*II gene excision from transgenic pollen and embryo

The T_1 progenies of eight selected self-pollinated T_0 plants were investigated for the presence of the *npt*II gene by germination and rooting assays. The scheme of the predicted genetic segregation upon self-pollination of a single-copy T_0 plant is given in Fig. 5a.

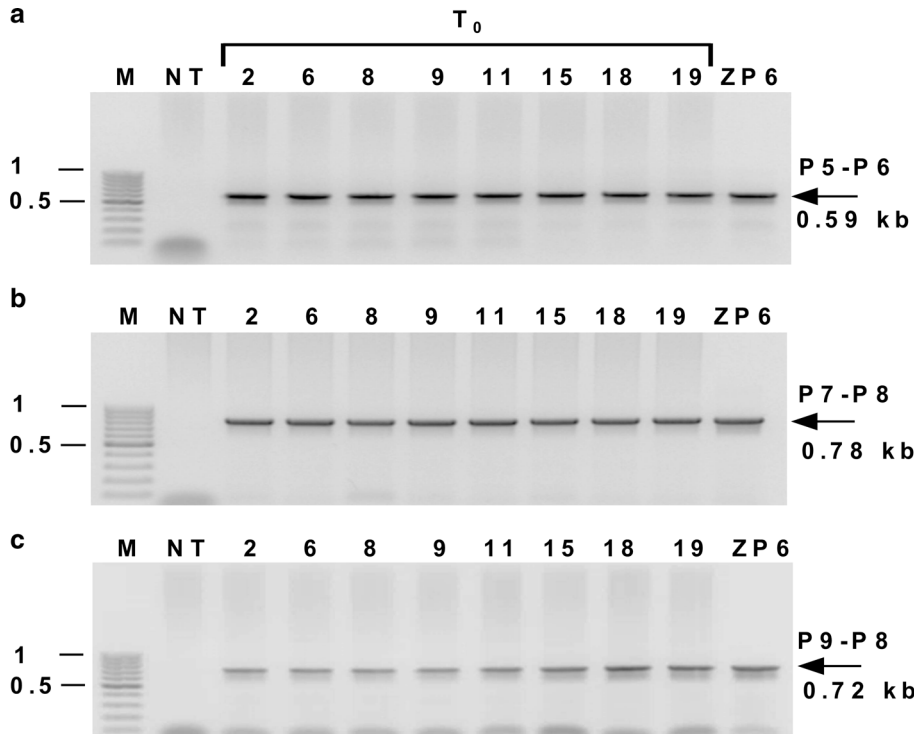

Fig. 2 Photographs of ethidium bromide-stained 1 % agarose gels with the PCR products obtained on transgenic T_0 plants. **a** PCR results with primers P5/P6 that amplified a 589 bp *gus-loxP-DLL* fragment. **b** PCR results with primers P7/P8 that amplified a 781 bp *nptII-loxP-RB end* fragment. **c** PCR results with primers P9/P8 that amplified a 716 bp *gus-DLL* fragment of the non-excised T-DNA. Lane M contains 100 bp DNA ladder (Fermentas) as a size marker, lanes 2–19 represent PCR products of individual transgenic T_0 plants, *NT* non-transformed plant, *ZP6* plasmid pZP6 used for plant transformation

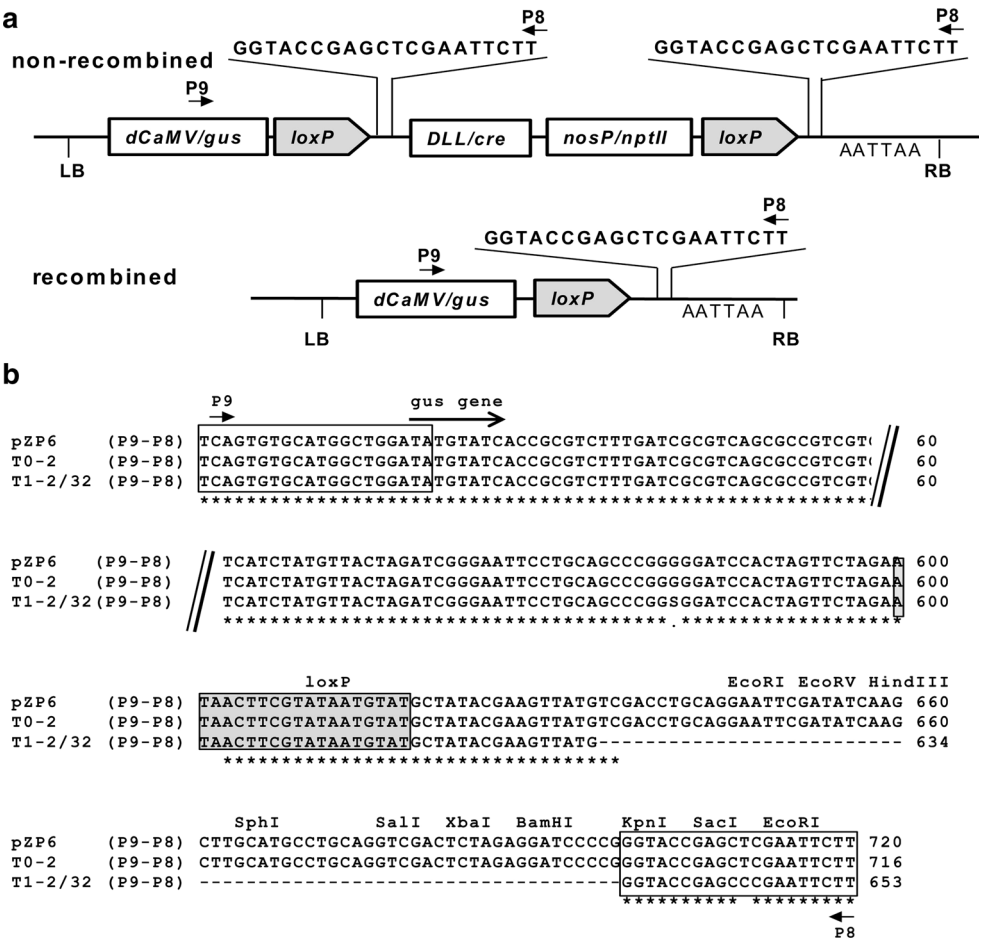

Fig. 3 The position of primers P9/P8 in the non- and recombined pZP6 T-DNA. **a** Using primers P9/P8 a 716-bp derived from the non-recombined T-DNA and a 653 bp from the recombined T-DNA PCR products could be generated. **b** Multiple alignment of the P9–P8 fragment from the transgenic tobacco line T_0-2 and the P9-P8 of T_1-2/32 derived from the self-pollinated line T_0-2 with the corresponding sequence of the plasmid pZP6. Alignment was generated using the CLUSTALW2 program. Nucleotides which are conserved in all aligned sequences are marked by *asterisk*. The *letters* in the *box* show the sequence of primers P9 and P8. *Dashes* show the *lox*P sequences

The number of seedlings sensitive to kanamycin (Km^S) ranged from 93.5 (T_0-2) to 100 % (T_0-15). These seedlings were also screened histochemically for GUS activity. The number of (GUS+, Km^S) seedlings ranged from 91.5 (T_0-2) to 99.0 % (T_0-9). The exception was line T_0-15 that produced progeny of GUS-positive seedlings that were all sensitive to kanamycin (Table 2; Table S1—Online resource 1).

In rooting assays, 8-week-old plants derived from seeds germinated under non-selective conditions had roots removed and were allowed to re-root in the presence of kanamycin. The proportion of T_1 plants that did not form any roots (R−) varied from 51.4 (T_0-6) to 89.0 % (T_0-9). None of the analysed T_0 lines produced completely (R−) T_1 plants. The number of (GUS+, R−) T_1 plants ranged from 17.0 % (T_0-6) to 86.4 % (T_0-9) (Table 2; Table S2—Online resource 1).

To study the excision of the *npt*II gene at molecular level, genomic DNAs from all phenotypically marker-free (GUS+, R−) T_1 plants were isolated and assayed by PCR. The analyses were carried out using the primer sets P1/P2, P9/P8 and P7/P8. PCR amplifications of a 744 bp P1–P2 fragment and a 781 bp P7–P8 fragment confirmed the presence of the *gus* gene and *nptII-loxP-RB end* sequences, respectively. An example of PCR is given in Fig. 6. As is shown in Fig. 3a, using the primer set P9/P8 could generate a 716 bp P9–P8 amplicon derived from the non-recombined and/or 653 bp P9–P8 amplicon generated from the recombined T-DNA.

The post-excision (653 bp) P9–P8 fragment was observed in 99.0 % (292 of 295) of T_1 plants (Table 3). Upon sequencing (T_1-2/32), the P9–P8 sequence was proven to be identical with the expected sequence (Fig. 3b). Based on the PCR results, the 295 analysed T_1 plants were divided into three groups (Table 3). Group A contains 241 plants that amplified both the P1–P2 and post-excision P9–P8 fragments, thus these plants carried the *gus* gene but not the *npt*II gene. These plants were considered marker-free. The number of marker-free T_1 plants varied between individual lines from 3.8 % (T_0-6) to 81.5 % (T_0-9). Group B comprises 51 plants that showed amplification of P1–P2, P7–P8 and both P9–P8 fragments. These T_1 plants were regarded as chimeric for the *npt*II gene. Group C includes three plants that showed amplification of P1–P2 and P9–P8 fragments (non-recombined T-DNA) and the P7–P8 fragment. The absence of a 653-bp P9–P8 fragment indicated no transgene excision because of a failure of the Cre/*lox*P system.

Taken together, Cre-mediated excision occurred in 292 out of 295 (groups A and B) analysed T_1 plants derived from eight self-pollinated T_0 lines. Of these, 241 plants were marker-free (group A) and 51 plants as were chimeric (group B).

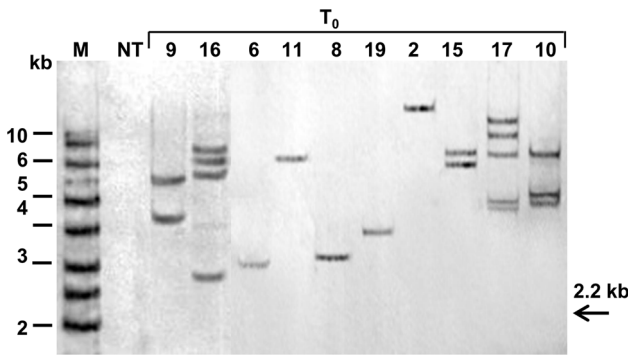

Fig. 4 Southern blot analyses on transgenic T_0 tobacco plants. KpnI-digested DNA was non-radioactively probed with the NPT-specific probe. The bands (>2.2 kb) correspond to the number of independent transgene copies. Lanes 2–19 represent individual transgenic T_0 plants. *NT* non-transformed plant

Efficiency of *npt*II gene excision from transgenic pollen

To investigate the excision of the *npt*II gene directly from transgenic pollen, pollen grains of selected T_0 lines (T_0-2, T_0-8, T_0-9, and T_0-19) were used to pollinate wild-type tobacco plants. The T_1 plants were first investigated for the presence/absence of the *npt*II gene by germination and rooting assays. The scheme of the predicted genetic segregation on cross-pollination of a single-copy T_0 plant is given in Fig. 5b.

In germination assays, the number of seedlings evaluated as kanamycin-sensitive was greater than 89 %. Two lines $WTxT_0$-9 and $WTxT_0$-19 produced progeny in which all histochemically GUS-positive seedlings were also kanamycin-sensitive (Table 4; Table S3—Online Resource 2). At the same time, the number of T_1 plants that did not form any roots (R−) ranged from 78.0 ($WTxT_0$-2) to 98.9 % ($WTxT_0$-9). None of the analysed T_0 lines produced completely (R−) T_1 plants. The number of (GUS+, R−) T_1 plants ranged from 48.4 ($WTxT_0$-19) to 98.1 % ($WTxT_0$-9) (Table 4; Table S4—Online Resource 2). These plants were considered putatively marker-free and further analysed.

In total, 137 (GUS+, R−) T_1 plants were subjected to PCR analyses with the primer sets P1/P2, P9/P8 and P7/P8. The post-excision P9–P8 fragment was detected in 99.3 % (136 of 137) analysed T_1 plants. As described above, the T_1 plants were divided into three groups. Data are summarised in Table 5. A total of 129 plants were sorted into group A and were considered marker-free. The number of marker-free plants varied between individual lines from 40.0 ($WTxT_0$-19) to 96.2 % ($WTxT_0$-9). Group B consisted of seven plants in which the *npt*II gene was only partially removed. These plants were chimeric for the *npt*II gene. Group C contained one plant in which the *npt*II gene was not excised because of a failure of the Cre/*lox*P system.

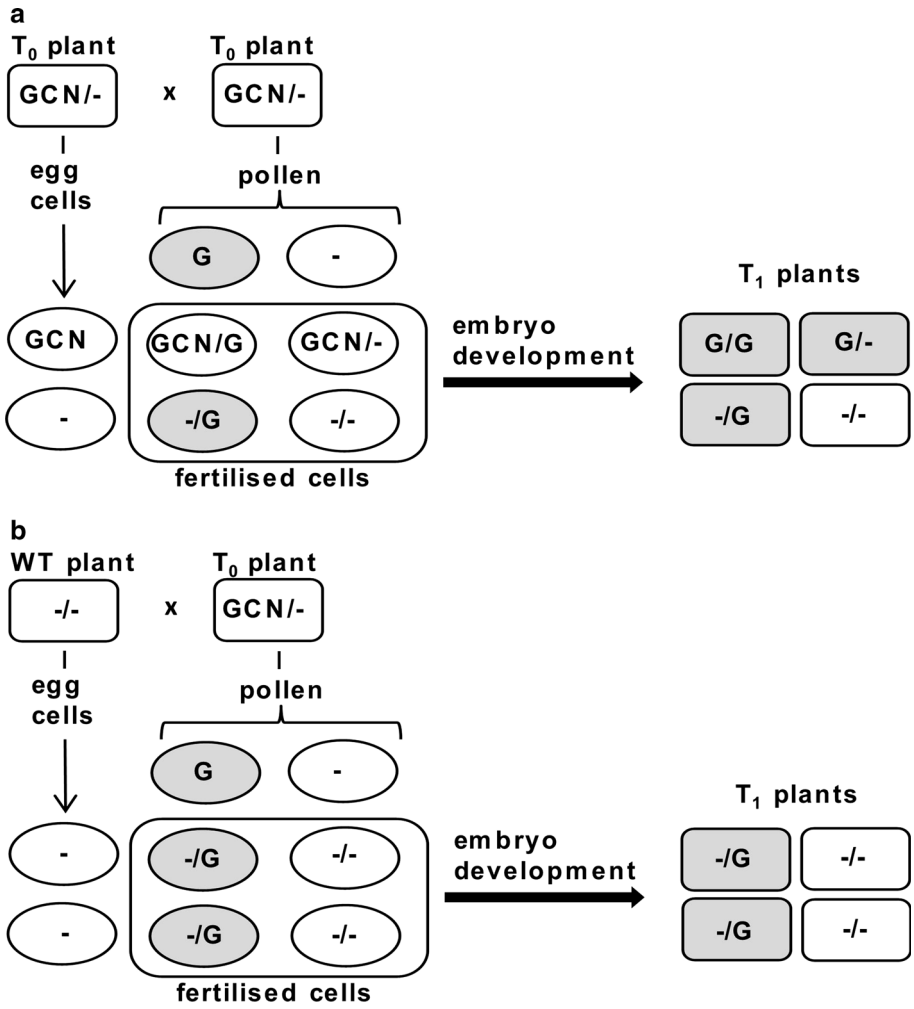

Fig. 5 Predicted genetic segregation of a single-copy transgenic T_0 plant upon **a** self-pollination and upon **b** crossing of a single-copy transgenic T_0 plant as a pollen donor to the wild-type (WT) plant. The scheme of Mlynarova et al. (2006) was adopted and modified. *G* β-Glucuronidase gene, *C cre* recombinase gene, *N* neomycin phosphotransferase gene

Taken together, Cre-mediated excision in pollen occurred in 136 of 137 T_1 plants analysed. There were 129 plants that were marker-free (group A) and seven plants were chimeric (group B).

Detailed analyses of the self- and cross-pollinated T_0-2 line

For a comprehensive view of excision events in transgenic progeny, both (GUS+, R−) (Tables 3, 5) and (GUS+, R+) T_1 plants from the self- and cross-pollinated line T_0-2 were analysed by PCR with the primer sets P1/P2, P9/P8, P7/P8. In all analysed (GUS+, R+) T_1 plants the P7–P8 fragment and both P9–P8 fragments were detected pointing to chimerism (Table 6). Excision occurred in all histochemically GUS-positive progenies of the self-pollinated T_0-2 line and in 98.0 % of plants derived from WTxT_0-2 line. However, only 46.4 and 49.0 % of self- and cross-pollinated plants were marker-free, respectively.

Discussion

The aim of this study was to evaluate the feasibility of the tissue-specific *Arabidopsis DLL* promoter in the Cre/*lox*P self-excision strategy. Corresponding gene characteristics (At4g16160, *Arabidopsis* eFP Browser database) with respect to cell types and expression level suggest its activation during pollen and embryo development but not in somatic cells. Indeed, recent work by Jopcik et al. (2014) showed no evidence for activation of *DLL* either in developing calli or in vegetative organs. Activity was detected strictly in pollen grains and tubes and in embryos at middle torpedo stage. Our work has confirmed that the *DLL* promoter is a strong candidate for use in the Cre/*lox*P self-excision strategy. Analyses of T_0 plants did not detect any premature excision of the *npt*II gene (Fig. 2c). This result is very encouraging since ectopic activation of another candidate, the *CRUC* promoter, has previously been shown to hamper its usability due to the unexpected promoter–enhancer interaction (Boszorádová et al. 2014).

Table 2 Segregation analyses of T_1 plants derived from the self-pollinated T_0 plants

Line[a]	GUS activity[b]	*Npt*II copy number[c]	Progeny							
			Germination assay[d]				Rooting assay[h]			
			Km^R:Km^S	Km^S (%)[e]	GUS+, Km^S [(f)]	GUS+, Km^S (%)[g]	R+:R−	R− (%)[i]	GUS+, R−[j]	GUS+, R− (%)[k]
T_0-2	48.49	1	9:129	93.5	97 (106)	91.5	32:57	64.0	37 (69)	53.6
T_0-6	67.13	1	1:130	99.2	66 (67)	98.5	44:47	51.6	9 (53)	17.0
T_0-8	106.23	1	1:111	99.1	83 (84)	98.8	11:72	86.7	53 (64)	82.8
T_0-11	90.80	1	4:102	96.2	71 (75)	94.7	17:71	80.7	49 (66)	74.2
T_0-19	43.77	1	4:115	96.6	73 (77)	94.8	37:63	63.0	20 (57)	35.1
T_0-9	86.60	2	1:116	99.1	96 (97)	99.0	11:89	89.0	70 (81)	86.4
T_0-15	101.69	2	0:126	100	99 (99)	100	23:76	76.8	47 (70)	67.1
T_0-18	75.40	2	1:107	99.1	51 (52)	98.1	38:54	58.7	10 (48)	20.8

[a] T_0 plants that were allowed to set the seeds after self-pollination

[b] GUS activity data of T_0 plants in picomoles of methylumbelliferone per minute per microgram of soluble protein

[c] The number of *npt*II gene copies estimated on the basis of Southern blot hybridisation with the non-radioactively labelled P3–P4 fragment as a probe

[d] Germination assays for kanamycin resistance. Seeds were germinated in the presence of 50 mg l^{-1} kanamycin, Km^R number of kanamycin-resistant seedlings, Km^S number of kanamycin-sensitive seedlings. The presence (GUS+) or absence (GUS−) of the GUS activity was determined histochemically

[e] The number of kanamycin-sensitive (Km^S) seedlings as a percentage of the total number of seedlings (Km^S, Km^R)

[f] The number of GUS-positive (GUS+) and kanamycin-sensitive (Km^S) seedlings. In brackets, the total number of (GUS+; Km^S, Km^R) seedlings is given

[g] The number of GUS-positive (GUS+) and kanamycin-sensitive (Km^S) seedlings as a percentage of the total number of (GUS+; Km^S, Km^R) seedlings

[h] Rooting assay for kanamycin resistance. Plants were allowed to root in the presence of 50 mg l^{-1} kanamycin, *R*+ number of rooted T_1 plants, *R*− number of non-rooted T_1 plants. The presence (GUS+) or absence (GUS−) of GUS activity was determined histochemically

[i] The number of (R−) plants as a percentage of the total number of (R+, R−) T_1 plants

[j] The number of GUS-positive and non-rooted (GUS+, R−) plants. In brackets, the total number of (GUS+; R+, R−) plants is given

[k] The number of GUS-positive and non-rooted (GUS+, R−) plants as a percentage of total number of (GUS+; R+, R−) T_1 plants

The ability of *DLL* to drive excision directly in pollen and embryos was further investigated in T_1 plants derived from eight self- and four cross-pollinated T_0 lines. In both types of pollination experiments, excision of the *npt*II gene was expected to result in sensitivity to kanamycin. However, in germination assays, there were kanamycin-sensitive as well as kanamycin-resistant seedlings detected in progenies of most self- and cross-pollinated T_0 lines (Tables 2, 4). Similarly, the results of rooting assays confirmed imperfect marker gene excision (Tables 2, 4). Nevertheless, the number of phenotypically marker-free T_1 plants was lower (Table 2). We assume that it could coincide with higher sensitivity of germinated seedlings to kanamycin. Probably, among the kanamycin-sensitive seedlings were also chimeric for the *npt*II gene with lower survival potential. In contrast, plants at later developmental stages, especially those chimeric for transgene, may withstand a certain level of selection agent, yet are unable to root. At the same time, the results of the rooting assays were consistent with the data obtained by PCR (Table 6).

In our previous self-excision experiments (Moravcikova et al. 2008) with the *CRUC* promoter active in developing embryos, excision efficiency was 10.2 % and an incomplete excision of the marker gene was found in most of the T_1 plants. In this study, the application of the promoter *DLL* resulted in up to 96.2 % marker-free plants (Tables 3, 5). Detailed analyses of the GUS-positive T_1 plants (self-pollinated T_0-2 line) showed that excision occurred in all plants, but chimeric plants were still detected (Table 6).

Since strong activity of *DLL* in a uninucleate microspore is assumed, only marker-free T_1 plants (cross-pollination) are theoretically expected (Fig. 5b). However, plants with incomplete excision of the *npt*II gene were also observed. We hypothesise that the activity of *DLL* in transgenic tobacco was shifted from the uninucleate microspore to a later stage of pollen development. It could coincide with a slightly different activity pattern of *DLL* in pollen of transgenic tobacco and *Arabidopsis* as an original organism. A delay in the transgene expression controlled by

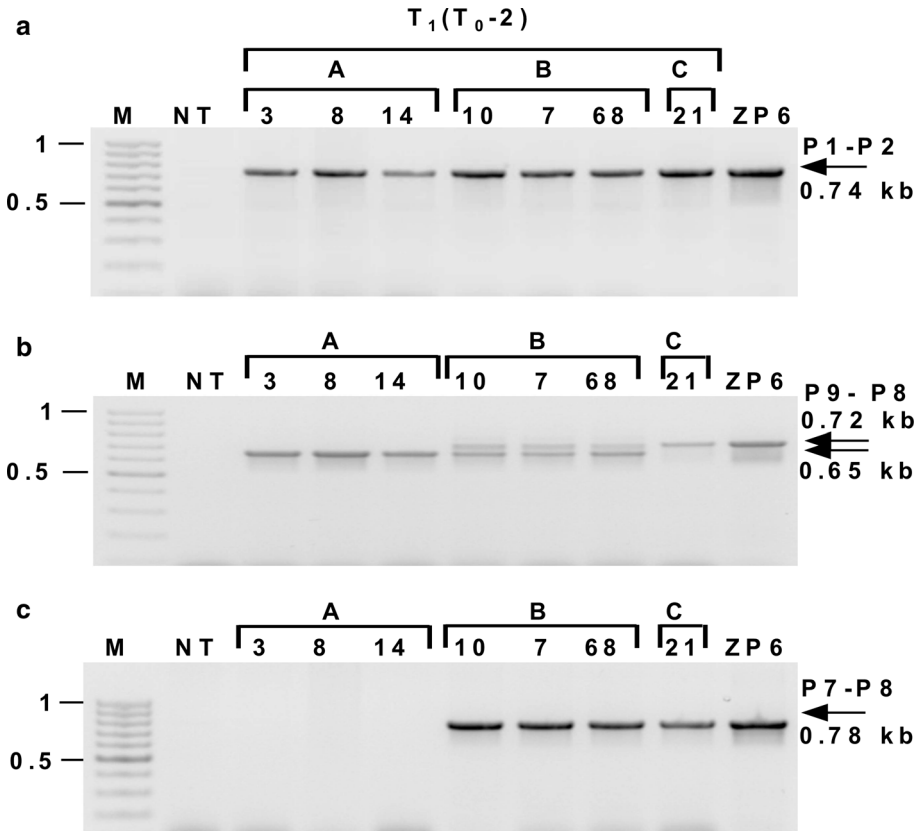

Fig. 6 Photographs of ethidium bromide-stained 1 % agarose gels with the PCR products obtained on tobacco T_1 plants. **a** PCR results with primers P1/P2 that amplified an internal 742-bp fragment of the *gus* gene. **b** PCR results with primers P9/P8 that amplified a 653-bp fragment derived from the recombined T-DNA and/or a 716-bp P9–P8 fragment derived from the non-recombined T-DNA. **c** PCR results with primers P7/P8 that amplified a 781 pb *nptII-loxP-RB end* fragment. Lane M contains 1 kb DNA ladder (Fermentas) as a size marker, lanes 3–21 represent PCR products of individual transgenic T_1 plants. The plants were sorted into three groups A, B and C. *NT* non-transformed tobacco plant, *ZP6* plasmid pZP6 used for plant transformation

Table 3 DNA analyses of T_1 plants derived from the self-pollinated T_0 lines

Line[a]	*Npt*II copy number[b]	PCR analyses of T_1 plants[d]				
		No. plants[c]	Group A Complete excision	Group B Chimeric	Group C No excision	Marker-free plants (%)[e]
T_0-2	1	37 (69)	32	5	0	46.4
T_0-6	1	9 (53)	2	5	2	3.8
T_0-8	1	53 (64)	42	11	0	65.6
T_0-11	1	49 (66)	46	3	0	69.7
T_0-19	1	20 (57)	11	9	0	19.3
T_0-9	2	70 (81)	66	4	0	81.5
T_0-15	2	47 (70)	37	10	0	52.9
T_0-18	2	10 (45)	5	4	1	4.4
Total		295 (505)	241	51	3	

[a] T_0 plants that were allowed to set the seeds after self-pollination

[b] The number of *npt*II gene copies estimated on the basis of Southern blot hybridisation with the non-radioactively labelled P3–P4 fragment as a probe

[c] The number of histochemically GUS-positive (GUS+) and non-rooted (R−) T_1 plants in the presence of 50 mg l^{-1} kanamycin that were subjected to PCR analyses. In brackets, the total number of GUS-positive T_1 plants is given

[d] Based on PCR results, plants were distributed into three groups: group A includes plants that showed amplification of P1–P2, P7–P8 and a 653-bp fragments but not a 716-bp P9–P8 fragment. Group B contains plants that showed amplification of P1–P2, P7–P8 and both 653 bp and a 716-bp P9–P8 fragments. Group C comprises plants that showed amplification P1–P2, P7–P8 and a 716-bp P9–P8 fragments but not a 653-bp P9–P8 fragment

[e] The number of T_1 plants with the completely removed *npt*II gene as a percentage of the total number of (GUS+; R+, R−) plants

Table 4 Segregation analyses of T_1 plants derived from the cross-pollinated T_0 lines

Line[a]	GUS activity[b]	Progeny								
		Germination assay[d]					Rooting assay[h]			
		*Npt*II copy number[c]	Km^R:Km^S	Km^S (%)[e]	GUS+, Km^S [(f)]	GUS+, Km^S (%)[g]	R+:R−	R− (%)[i]	GUS+, R−[j]	GUS+, R− (%)[k]
$WTxT_0$-2	48.49	1	11:94	89.5	38 (48)	79.2	22:78	78.0	27 (49)	55.1
$WTxT_0$-8	106.23	1	1:115	99.1	58 (59)	98.3	4:76	95.9	43 (47)	91.5
$WTxT_0$-19	43.77	1	0:125	100	66 (66)	100	16:83	83.8	15 (31)	48.4
$WTxT_0$-9	86.60	2	0:108	100	66 (66)	100	1:93	98.9	52 (53)	98.1

[a] T_0 plants that were allowed to set the seeds after self-pollination

[b] GUS activity data of T_0 plants in picomoles of methylumbelliferone per minute per microgram of soluble protein

[c] The number of *npt*II gene copies estimated on the basis of Southern blot hybridisation with the non-radioactively labelled P3-P4 fragment as a probe

[d] Germination assays for kanamycin resistance. Seeds were germinated in the presence of 50 mg l^{-1} kanamycin, Km^R number of kanamycin-resistant seedlings, Km^S number of kanamycin-sensitive seedlings. The presence (GUS+) or absence (GUS−) of the GUS activity was determined histochemically

[e] The number of kanamycin-sensitive (Km^S) seedlings as a percentage of the total number of seedlings (Km^S, Km^R)

[f] The number of GUS-positive (GUS +) and kanamycin-sensitive (Km^S) seedlings. In brackets, the total number of (GUS+; Km^S + Km^R) seedlings is given

[g] The number of GUS-positive (GUS+) and kanamycin-sensitive (Km^S) seedlings as a percentage of the total number of (GUS+; Km^S + Km^R) seedlings

[h] Rooting assay for kanamycin resistance. Plants were allowed to root in the presence of 50 mg l^{-1} kanamycin, *R*+ number of rooted T_1 plants, *R*− number of non-rooted T_1 plants. The presence (GUS+) or absence (GUS−) of GUS activity was determined histochemically

[i] The number of (R−) plants as a percentage of the total number of (R+, R−) T_1 plants

[j] The number of GUS-positive and non-rooted (GUS+, R−) plants. In brackets, the total number of (GUS+; R+, R−) plants is given

[k] The number of GUS-positive and non-rooted plants (GUS+, R−) as a percentage of total number of (GUS+; R+, R−) T_1 plants

Table 5 DNA analyses of T_1 plants derived from the cross-pollinated T_0 lines

Line[a]	*Npt*II copy number[b]	PCR analyses of T_1 plants[d]				Marker-free plants (%)[e]
		No. plants[c]	Group A *Npt*II gene excision	Group B Chimeric	Group C without excision	
$WTxT_0$-2	1	27 (49)	24	2	1	49.0
$WTxT_0$-8	1	43 (47)	42	1	0	89.4
$WTxT_0$-19	1	15 (30)	12	3	0	40.0
$WTxT_0$-9	2	52 (53)	51	1	0	96.2
	Total	137 (179)	129	7	1	

[a] T_0 plants were pollen donors and wild-type (WT) plants were seeds parents for these crosses

[b] The number of *npt*II gene copies estimated on the basis of Southern blot hybridisation with the non-radioactively labelled P3–P4 fragment as a probe

[c] The number of histochemically GUS-positive (GUS+) and non-rooted (R−) T_1 plants in the presence of 50 mg l^{-1} kanamycin that were subjected to PCR analyses. In brackets, the total number of GUS-positive T_1 plants is given

[d] Based on PCR results, plants were distributed into three groups: group A includes plants that showed amplification of P1–P2, P7–P8 and a 653-bp fragments but not a 716-bp P9–P8 fragment. Group B contains plants that showed amplification of P1–P2, P7–P8 fragments and both 653-bp and 716-bp P9–P8 fragments. Group C comprises plants that showed amplification of P1–P2, P7–P8 and a 716-bp P9–P8 fragments but not a 653-bp P9–P8 fragment

[e] The number of T_1 plants with the completely removed *npt*II gene as a percentage of the total number of (GUS+; R+, R−) plants

tissue-specific promoters has been reported (Odell et al. 1994; Jopcik et al. 2014).

The relatively high number of marker-free plants is likely to be a result of the *cre* expression in pollen rather than in both pollen and embryo. For example, the self-pollinated line T_0-8 generated marker-free T_1 plants with efficiency of 65.6 % (Table 3). When the line T_0-8 was used as a pollen donor to a wild type, 89.4 % excision

Table 6 Detailed DNA analyses of T_1 plants derived from the self- and cross-pollinated line T_0-2

T_0 plants			PCR analyses of T_1 plants[c]			
Line[a]		No. plants[b]	Group A Complete excision	Group B Chimeric	Group C No excision	Excision event (%)[d]
T_0-2	(GUS+, R−)	37	32 (46.4 %)	5	0	
(selfing)	(GUS+, R+)	32	0	32	0	
	Total	69	32	37	0	100 %
WTxT_0-2	(GUS+, R−)	27	24 (49.0 %)	2	1	
(crossing)	(GUS+, R+)	22	0	22	0	
	Total	49	24	24	1	98.0 %

[a] The self- and cross-pollinated line T_0-2. In cross-pollination experiments, the line T_0-2 was used as pollen donor to the wild type (WT)

[b] The number of analysed T_1 plants. (GUS+, R−) histochemically GUS-positive plants that did not form roots in the presence of 50 mg l^{-1} kanamycin, (GUS+, R+) histochemically GUS-positive plants that form roots in the presence of 50 mg l^{-1}

[c] Based on PCR results, plants were distributed into three groups: group A includes plants that showed amplification of a P1–P2, P7–P8 and a 653-bp fragments but not a 716-bp P9–P8 fragment. Group B contains plants that showed amplification of a P1–P2, P7–P8 fragments and both 653 and 716-bp P9–P8 fragments. Group C comprises plants that showed amplification of a P1–P2, P7–P8 and a 716-bp P9–P8 fragments but not a 653-bp P9–P8 fragment. In brackets, the number of marker-free T_1 plants as a percentage of the total number of (GUS+; R+, R−) plants is given

[d] The number of T_1 plants that amplified the post-excision P9–P8 fragment as a percentage of the total number of (GUS+; R+, R−) plants

efficiency was achieved (Table 5). We assume this phenomenon is a result of non-uniform expression of the *cre* gene in an embryo. An embryo represents a more complex multicellular structure than bicellular pollen. To gain marker-free plants, the *cre* gene has to be sufficiently expressed in every embryogenic cell. A relationship between the *cre* expression level and recombination efficiency has been found in other studies (Marjanac et al. 2008).

In summary, marker-free plants were obtained from all transgenic tobacco lines with an excision efficiency up to 96.2 %, depending on the type of pollination experiment (Tables 3, 5). At the same time, differences in excision efficiency among individual transgenic lines likely resulted from the position effect of the transgene insertion in the tobacco genome (Matzke and Matzke 1998).

Most previously reported self-excision recombination systems have relied mainly on the activity of embryo-specific promoters (Li et al. 2007; Moravcikova et al. 2008; Kopertekh et al. 2009; Chong-Perez et al. 2013). To date, only few pollen- and embryo-specific promoters have been investigated (Luo et al. 2007). This study adds the *DLL* promoter to the list of candidates for efficient generation of marker-free plants.

Our study has proved the feasibility of the pollen- and embryo-specific *DLL* promoter to generate marker-free T_1 plants. Our results using tobacco as a model plant are promising for potential use in other sexually propagated plant species. Marker-free transgenic plants generated in this way can contribute to biosafety and greater acceptability of GM plants to the public.

Author contribution statement JL, IM and JM designed the entire experiments. The experiments were carried out by ZP, MJ, JL and JM. JM and IM wrote and edited the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest The authors declare they have no conflict of interest.

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