

Evaluation of CRE-mediated excision approaches in *Arabidopsis thaliana*

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Abstract The ability of the CRE recombinase to catalyze excision of a DNA fragment flanked by directly repeated *lox* sites has been exploited to modify gene expression and proved to function well in particular case studies. However, very often variability in *CRE* expression and differences in efficiency of CRE-mediated recombination are observed. Here, various approaches were investigated to reproducibly obtain optimal CRE activity. CRE recombination was analyzed either by transforming the CRE T-DNA into plants containing a *lox*-flanked fragment or by transforming a T-DNA harboring a *lox*-flanked fragment into plants producing the CRE

recombinase. Although somatic CRE-mediated excision of a *lox*-flanked fragment was obtained in all transformants, a variable amount of germline-transmitted deletions was found among different independent transformants, irrespective of the orientation of transformation. Also, the efficiency of CRE-mediated excision correlated well with the *CRE* mRNA level. In addition, CRE-mediated fragment excision was compared after floral dip and after root tissue transformation when transforming in a *CRE*-expressing background. Importantly, less CRE activity was needed to excise the *lox*-flanked fragment from the transferred T-DNA after root tissue transformation than after floral dip transformation. We hypothesize that this is correlated with the lower T-DNA copy number inserted during root transformation as compared to floral dip transformation.

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Introduction

Several site-specific recombination systems from bacteriophages and yeast have been well characterized (Odell and Russell 1994; Ow 2002, Thomson and Ow 2006) and found to be functional in a wide range of organisms. The most widely used in plants is the bacteriophage P1 CRE/*loxP* site-specific

recombination system (Gilbertson 2003). This CRE recombinase system has been exploited extensively to remove selectable marker genes (Dale and Ow 1990; Russell et al. 1992; Zuo et al. 2001; Hoa et al. 2002; Zhang et al. 2003; Sreekala et al. 2005). Another application is the resolution of complex transgene loci to single copies after direct gene transfer (Srivastava et al. 1999; Srivastava and Ow 2001). In addition, the CRE/*lox* recombination system for targeted DNA insertion proved to be efficient when DNA was introduced by particle bombardment (Srivastava et al. 2004; Chawla et al. 2006), but was ineffective either after polyethylene glycol-mediated protoplast transformation because of high rates of gene silencing or after *Agrobacterium*-mediated transformation because of low transformation frequencies (Vergunst and Hooykaas 1998; Vergunst et al. 1998; Day et al. 2000). In the above described examples, the *lox* constructs and the *CRE* gene were combined either by retransformation or by genetic crossings. The timing of gene expression can be highly controlled by using inducible promoters, such as promoters regulated by chemicals or heat shock for switching on CRE at the desired time point (Hoff et al. 2001; Joubès et al. 2004; Sreekala et al. 2005; Wang et al. 2005). Furthermore, strategies for transient expression of the CRE recombinase in plants have been successful either by cocultivation with recombinant *Agrobacterium* strains (Gleave et al. 1999), by use of an inducible self-excising *CRE* expression vector (Zuo et al. 2001), or by applying plant virus vector systems (Kopertekh et al. 2004; Jia et al. 2006).

Several reports have been published on variations in excision efficiency (Odell et al. 1990; Bayley et al. 1992; Russell et al. 1992; Hoa et al. 2002) that have been postulated to be caused by the chromosomal position of the transgenic loci and/or the difference in CRE recombinase activity in the *lox* target locus and *CRE*-expressing locus. The efficiency of recombination seemed to be related primarily to the CRE parent and has been hypothesized to be most probably due to different *CRE* expression levels in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Russell et al. 1992). In addition, the excision efficiency was much higher when the *lox* target plant was transformed with a *CRE*-containing T-DNA than when *CRE*-expressing plants were crossed with *lox* target plants, indicating that various degrees of excision efficiency

are obtained with different combination methods for the *lox*-flanked gene and the CRE recombinase (Russell et al. 1992). The above examples illustrate that, although CRE-mediated excision can work reliably, many problems are observed with the reproducibility.

The objective of this work was to obtain a clear view on the effectiveness of CRE-mediated excision in *A. thaliana*. In particular, the question was asked whether CRE-mediated recombination is more efficient when the *lox*-flanked target is transformed into a CRE background or vice versa when a *CRE*-expressing T-DNA construct is transformed into a *lox*-flanked target fragment-containing plant. Therefore, firstly, we transformed a *CRE*-expressing T-DNA into plants containing a *loxP*-flanked β -glucuronidase (*GUS*)-coding cassette and, secondly, a T-DNA with the *loxP*-flanked *GUS* cassette into two different homozygous *CRE*-expressing plants, either by floral dip transformation or by root explant cocultivation, allowing the CRE recombination activity to be compared in both series of transformants. By using different *CRE*-expressing plants, a possible direct correlation between the CRE recombination efficiency and the *CRE* expression level could be analyzed.

Results

Strategy to evaluate CRE-mediated excision of the *GUS* expression cassette

The presence or absence of the *GUS* gene between two tandemly repeated *loxP* sites was used as reporter system for CRE-mediated recombination (Fig. 1a, b) (De Buck et al. 2000). The K2L T-DNA contains a kanamycin resistance gene as selectable marker and a 35S-driven *GUS* gene flanked by two directly repeated *loxP* sites (Fig. 1a) (De Buck et al. 1998). The chosen CK2L6 line of *Arabidopsis* is homozygous for the transgenic locus containing a single K2L T-DNA copy inserted into chromosome 5 and displays stable and high *GUS* expression in the F2 generation (Theuns et al. 2002, De Buck et al. 2004). The excision of the *GUS* gene by introduction of CRE was analyzed by histochemical staining and by polymerase chain reaction (PCR) analysis (Fig. 1a, b; Materials and methods).

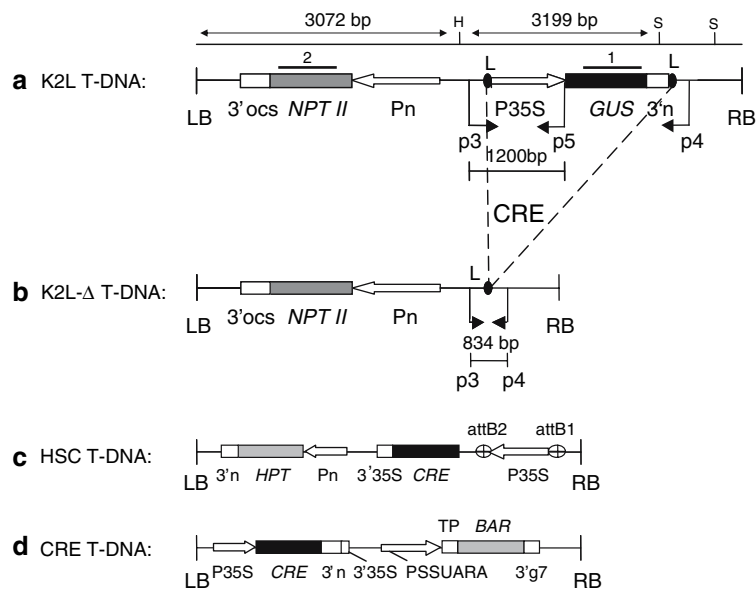


Fig. 1 Strategy for CRE-mediated excision of the *GUS* expression cassette. **(a)** Schematic representation of the K2L T-DNA in the CK2L6 line. The K2L T-DNA consists of a kanamycin selectable marker and *P35S-GUS-3'n* expression cassette (not on scale). After digestion of the plant DNA with *Hind*III and *Sac*I, a *GUS* fragment of 3199 bp will be observed after hybridization with the *GUS* probe (probe 1) when the *GUS* gene is present in the genome and a T-DNA/plant junction fragment of at least 3072 bp will be observed after hybridization with the *NPTII* probe (probe 2). **(b)** The derived K2L- Δ T-DNA after CRE-mediated recombination of the directly oriented *loxP* sites (L), resulting from the excision of the *GUS* expression cassette. **(c)** Schematic outline of the HSC T-DNA in pHsc. **(d)** Schematic outline of the CRE T-DNA in

pCre. Abbreviations: 3'ocs, 3' end of the octopine synthase gene; *NPTII*, neomycin phosphotransferase II gene; Pn, promoter of the nopaline synthase gene; L, *loxP* sequence recognition site of the CRE/*loxP* recombination system; P35S, cauliflower mosaic virus promoter; *GUS*, β -glucuronidase-coding sequence; *HPT*, hygromycin phosphotransferase gene; 3'n, 3' end of the nopaline synthase gene; 3'35S, 35S terminator; *CRE*, CRE recombinase gene; *BAR*, phosphinothricin transferase gene; *PSSUARA*, the small subunit promoter of *Arabidopsis*; TP, transit peptide; 3'g7, 3' terminator of gene 7; attB1 and attB2, GATEWAYTM-compatible recombination sites; H, *Hind*III; S, *Sac*I; LB, left border; RB, right border; p3, p4, and p5, primers used for PCR analysis

Efficiency of CRE-mediated excision of the *GUS* expression cassette by transformation of a *P35S-CRE* T-DNA into CK2L6 *Arabidopsis* plants

In order to analyze the effectiveness of CRE recombinase to induce DNA recombination and excision of the *GUS* gene, CK2L6 *Arabidopsis* plants were retransformed by the floral dip method with the HSC T-DNA (Fig. 1c) that contains a P35S-driven *CRE* gene and a hygromycin-selectable marker gene (Materials and Methods). T2 progeny plants of ten independent hygromycin-resistant CK2L6-SC retransformants were stained for *GUS* activity (Table 1). All T2 seedlings derived from retransformant CK2L6-SC13 were *GUS*-negative (Fig. 2T) implying that the CRE recombinase excised the *GUS* cassette

during the T1 generation. Among the progeny of the other nine CK2L6-SC retransformants, a variable number of *GUS*-negative and *GUS*-positive seedlings with a mosaic pattern were observed (Table 1). The mosaic pattern resulted from *GUS* gene excision in parts of the cells of the growing T2 progeny plants, implying inheritance of a non-excised form of the K2L T-DNA from the T1 CK2L6-SC retransformants. For example, eight of ten T2 seedlings of retransformant CK2L6-SC6 were *GUS*-negative and only two had a patchy *GUS* activity distribution, mainly in the hypocotyl and primary root cells (Fig. 2G, H, I). In retransformant CK2L6-SC3, seven T2 seedlings were *GUS*-negative and in the remaining three *GUS* was active in the root hairs of the primary root (Fig. 2B). Furthermore, in the CK2L6-SC5, CK2L6-SC7, CK2L6-SC9, CK2L6-SC10, and

Table 1 GUS staining of T2 progeny of CK2L6 plants re-transformed by the floral dip method with a *CRE*-expressing T-DNA

Transformant	T2 seedlings	GUS-positive ^a	GUS-negative
CK2L6-SC3	10	3	7
CK2L6-SC4	10	8	2
CK2L6-SC5	10	5	5
CK2L6-SC6	10	2	8
CK2L6-SC7	10	5	5
CK2L6-SC9	10	4	6
CK2L6-SC10	10	6	4
CK2L6-SC11	10	8	2
CK2L6-SC12	10	5	5
CK2L6-SC13	10	0	10

^a Refers to a mosaic staining pattern

CK2L6-SC12 retransformants, four to six of the progeny plants displayed a patchy distribution of GUS activity in the cotyledons, hypocotyls, and the primary root cells (Fig. 2 CK2L6-SC5 (E, F);

CK2L6-SC7 (J, K); CK2L6-SC9 (L, M); CK2L6-SC10 (N, O); CK2L6-SC12 (R, S)). Finally, in transformants CK2L6-SC4 and CK2L6-SC11, only two of the T2 seedlings were GUS-negative, while the remaining eight seedlings had large GUS-positive sectors in the cotyledons, hypocotyls, primary roots, and in some lateral roots (Fig. 2 CK2L6-SC4 (C, D); CK2L6-SC11 (P, Q)).

To rule out that silencing was the reason for the absence of GUS staining rather than *CRE*-mediated *GUS* excision, a molecular analysis was performed with the primer sets p3 + p4 and p3 + p5 (Fig. 1). For the T2 seedlings from the transformant CK2L6-SC13, only a 834 bp fragment was detected, indicating efficient *CRE*-mediated excision of the *GUS* gene in the analyzed leaves (Fig. 3). This result corresponds with the histochemical staining because no GUS activity was observed in any of the T2 progeny plants (Fig. 2T). The progeny from the other nine CK2L6-SC retransformants showed a signal for both the 834-bp fragment and the 1200-bp fragment, indicative for the

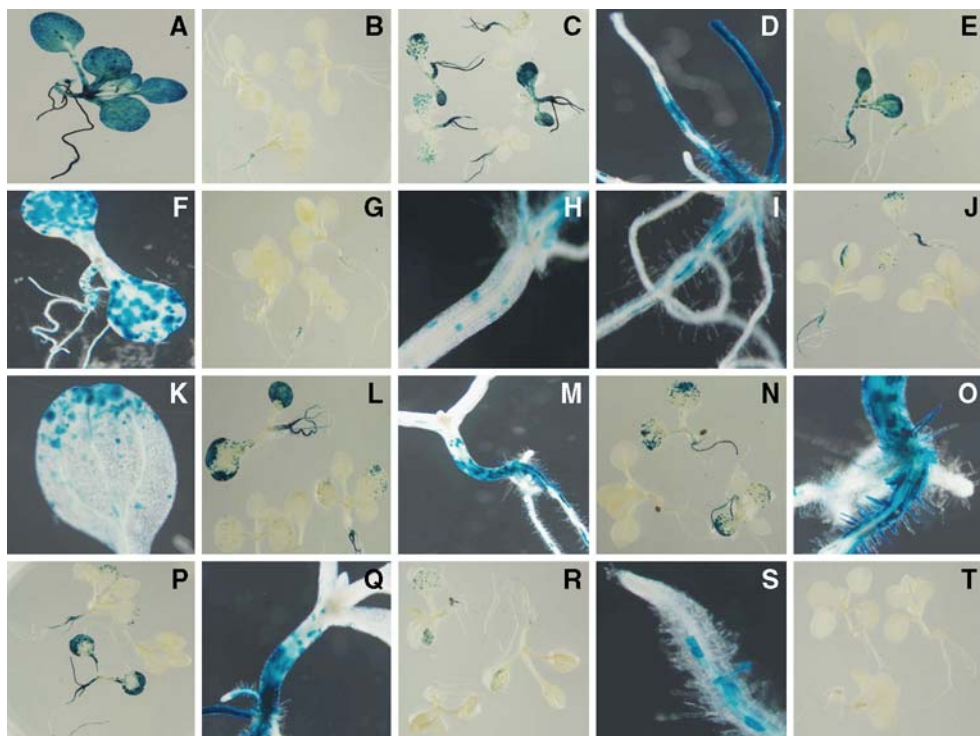


Fig. 2 Histochemical GUS staining of 2-week-old seedlings. Histochemical analysis of T2 progeny from ten independent CK2L-SC retransformants. CK2L6 parental line (A); representative examples of T2 progeny plants from transformant CK2L6-SC3 (B), CK2L6-SC4 (C, D), CK2L6-SC5 (E, F),

CK2L6-SC6 (G, H, I), CK2L6-SC7 (J, K), CK2L6-SC9 (L, M), CK2L6-SC10 (N, O), CK2L6-SC11 (P, Q), CK2L6-SC12 (R, S), and CK2L6-SC13 (T). Seedlings were grown for two weeks on K1 medium supplemented with 20 mg/l hygromycin

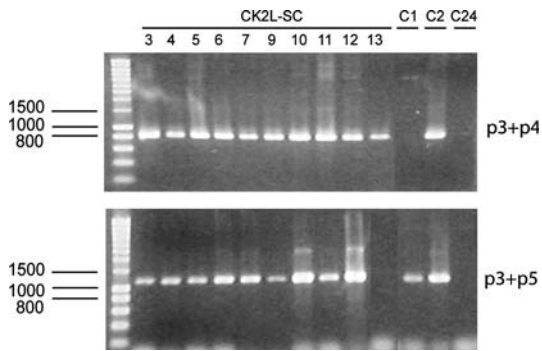


Fig. 3 PCR analysis of genomic DNA prepared from T2 progeny from the ten independent CK2L6-SC retransformants. Primers 3, 4, and 5 were used as illustrated in Fig. 1. Seedlings were grown for two weeks on K1 medium supplemented with 20 mg/l hygromycin. The following controls were added: C1, CK2L plant; C2, KH15d plant (deletion line containing both the *GUS* expression cassette and the deletion [De Buck et al. 2001]), and C24 wild-type DNA

presence of the *GUS* cassette, correlating with the mosaic *GUS* staining pattern (Fig. 3).

To investigate whether the observed differential CRE-mediated recombination efficiency could be caused by a difference in *CRE* expression levels, we performed a real-time quantitative PCR analysis. The retransformants CK2L6-SC11 and CK2L6-SC13 were chosen as representatives for low and high rates of *GUS* excision, respectively (Fig. 4). *CRE* mRNA levels in the CK2L6-SC13 retransformant were five-fold higher than those in the CK2L6-SC11 retransformant, corresponding with the *GUS* staining results of T2 progeny plants. This difference indicates that high *CRE* mRNA levels are required to obtain high rates of CRE-mediated recombination.

In conclusion, although all ten CK2L6-SC retransformants showed functional CRE activity, the efficiency of CRE-mediated excision of the *GUS* expression cassette varied significantly between the different CK2L6-SC retransformants. Furthermore, we demonstrated a correlation between the *CRE* mRNA expression level and excision efficiency in the CK2L6-SC11 and the CK2L6-SC13 transformants.

Efficiency of CRE-mediated excision by transformation of the *lox-GUS* K2L T-DNA into *CRE*-expressing *Arabidopsis* plants

Two homozygous, single-locus *CRE*-expressing lines, Cre1 and Cre13 (Materials and Methods)

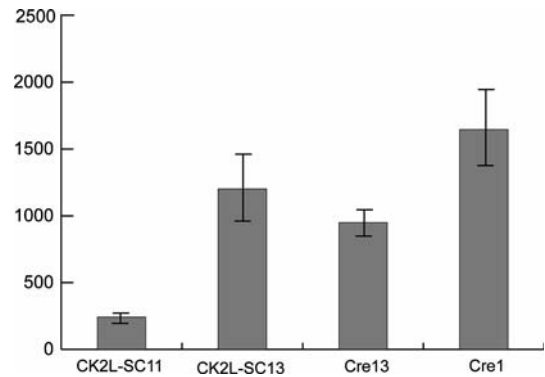


Fig. 4 Expression analysis of *P35S-CRE* in the CK2L6-SC11, CK2L6-SC13, Cre1, and Cre13 lines. mRNA accumulation levels in pooled 2-week-old seedlings were determined by real-time PCR with *ACTIN2* as constitutive control. The fold difference of expression in the analyzed lines compared to the untransformed control is shown. Error bars are standard deviation of two biological repeats. Seedlings were grown for two weeks on K1 medium supplemented with 20 mg/l hygromycin (CK2L6-SC11 and CK2L6-SC13) or non-selective K1 medium (Cre1 and Cre13)

containing the CRE T-DNA (De Buck et al. 2000) (Fig. 1d), were used to determine whether the CRE-mediated recombination can be obtained with a higher frequency when the target is transformed into *CRE*-expressing background (see above). Therefore, Cre1 and Cre13 plants were retransformed with the *lox* target-containing K2L T-DNA by floral dip transformation. Four out of six T2 progenies of the retransformants into Cre1 background (FC1K2L) were *GUS*-negative, but none of the T2 progenies of the ten analyzed retransformants into Cre13 background (FC13K2L) showed excision of the *GUS* cassette in all progeny plants (Table 2). Moreover, the T2 plants of FC1K2L-2-3, which still contained the *GUS* gene were only weakly stained, while seedlings of all FC13K2L T2 plants displayed a mosaic pattern ranging from minimally to completely *GUS*-stained (data not shown). Thus, the retransformed Cre1 and the Cre13 plants differed clearly in CRE efficiency for *GUS* excision from the K2L T-DNA. The PCR results fully corroborated the *GUS* staining pattern for all the transformants, meaning that a mosaic *GUS* pattern correlated with the amplification of the 1200-bp fragment that is diagnostic for the presence of the *GUS* cassette (Table 2). In the absence of *GUS* staining, only the diagnostic fragment of 834 bp was found indicative of the K2L T-DNA excision allele (Table 2).

Table 2 GUS staining and PCRs of T2 progeny of Cre1 and Cre13 plants retransformed by the floral dip method with the K2L T-DNA vector

Transformant	T2 seedlings	GUS-positive ^a	GUS-negative	p3 + p4 ^b 834 bp	p3 + p5 ^b 1200 bp
<i>Cre1 retransformants (FC1K2L)</i>					
FC1K2L-2-3	5	3	2	+	+
FC1K2L-3-1	5	0	5	+	–
FC1K2L-3-5	5	0	5	+	–
FC1K2L-3-3	5	0	5	+	–
FC1K2L-3-7	5	0	5	+	–
FC1K2L-3-4	5	5	0	+	+
<i>Cre13 retransformants (FC13K2L)</i>					
FC13K2L-1-4	5	5	0	+	+
FC13K2L-4-1	5	5	0	+	+
FC13K2L-3-2	5	5	0	+	+
FC13K2L-1-1	5	5	0	+	+
FC13K2L-1-3	5	5	0	+	+
FC13K2L-2-2	5	5	0	+	+
FC13K2L-2-1	5	5	0	+	+
FC13K2L-3-3	5	5	0	+	+
FC13K2L-1-5	5	5	0	+	+
FC13K2L-3-4	5	5	0	+	+

^a Refers to a mosaic staining pattern

^b Primers used for PCR, see Fig. 1

Real-time PCR analysis revealed that the *CRE* mRNA level was approximately two-fold higher in the Cre1 plants than in the Cre13 plants, which is in agreement with the observed different rate of CRE-mediated recombination (Fig. 4).

The K2L T-DNA was also introduced into Cre1 and Cre13 plants via root explant transformation. T2 seedlings of nine retransformants in Cre1 (RC1K2L) and of eight retransformants in Cre13 (RC13K2L) were analyzed by GUS staining and PCR analysis (Table 3; Materials and Methods). Strikingly, none of the T2 progenies of the RC1K2L retransformants still contained the *GUS* gene. Also, the *GUS* cassette was excised in all of the T2 progeny plants of seven of the eight RC13K2L retransformants.

To explain the higher rate of *GUS* excision after root transformation than after floral dip transformation, *GUS* excision and T-DNA integration pattern for 1 FC1K2L, 5 FC13K2L, 5 RC1K2L, and 3 RC13K2L retransformants were determined by DNA gel blot analysis (Fig. 5). The *HindIII/SacI* digest was chosen to allow detection of the internal 3199-bp *GUS* fragment with the *GUS* probe and the

visualization of the left T-DNA-plant junction fragment with the neomycin phosphotransferase II (NPTII) probe (Fig. 1). The obtained results with the *GUS* probe (Fig. 5a) fully confirmed the PCR analysis and GUS staining pattern. For retransformants FC1K2L3-3, no *GUS* fragment could be detected, while for all the tested FC13K2L retransformants the *GUS* fragment was present. In all RC1K2L and RC13K2L retransformants, the 3199-bp *GUS* fragment was absent, except in RC13K2L-27a where it could be visualized analogously to the PCR and GUS staining results. The presence of the other hybridization bands with the *GUS* probe is probably caused by partial digestion because of methylation of the *HindIII-SacI* restriction sites (Nelson et al. 1993). Moreover, inverted repeats of additional T-DNAs in the locus may cause inversion of the segments between two inverted *loxP* sites and result in new *GUS* fragments. Hybridization with the NPTII probe indicated that on average more T-DNA copies were integrated in floral dip than in root tissue transformants (Fig. 5b). On average, more than two LB/plant border junctions were observed for the floral dip

Table 3 GUS staining and PCRs of T2 progeny of Cre1 and Cre13 plants transformed by the root transformation method with the K2L T-DNA vector

Transformant	T2 seedlings	GUS-positive ^a	GUS-negative	p3 + p4 ^b 834 bp	p3 + p5 ^b 1200 bp
<i>Cre1 retransformants (RC1K2L)</i>					
RC1K2L-51	5	0	5	+	–
RC1K2L-3-51	5	0	5	+	–
RC1K2L-3a	5	0	5	+	–
RC1K2L-18a	5	0	5	+	–
RC1K2L-73	5	0	5	+	–
RC1K2L-5b	5	0	5	+	–
RC1K2L-8a	5	0	5	+	–
RC1K2L-19c	5	0	5	+	–
RC1K2L-26	5	0	5	+	–
<i>Cre13 retransformants (RC13K2L)</i>					
RC13K2L-36	5	0	5	+	–
RC13K2L-35	5	0	5	+	–
RC13K2L-61	5	0	5	+	–
RC13K2L-22	5	0	5	+	–
RC13K2L-2a	5	0	5	+	–
RC13K2L-5b	5	0	5	+	–
RC13K2L-39	5	0	5	+	–
RC13K2L-27a	5	5	0	+	+

^a Refers to a mosaic staining pattern

^b Primers used for PCR, see Fig. 1

retransformants, while retransformants obtained by root transformation contained on average one or two LB/plant border junctions.

All together, we demonstrated that when a *loxP*-containing T-DNA was transformed into *CRE*-expressing plants, the efficiency of *CRE*-mediated excision varied according to the *CRE*-expressing line, indicating that the *CRE* expression level is most probably the most discriminating factor to obtain high *CRE* activity. Moreover, our results convincingly show that *GUS* excision from the introduced K2L T-DNA was much more efficient after root explant than after floral dip transformation.

Discussion

We analyzed the *CRE*-mediated excision efficiency of a *loxP*-flanked *GUS* expression cassette in transgenic *Arabidopsis* plants after transformation of the *CRE* construct into the target plant and after transformation of the target into *CRE*-expressing plants.

For this latter approach, both the floral dip and the more labour-intensive root explant transformation method were compared.

Upon floral dip transformation of CK2L6 plants with the *P35S-CRE* construct, a variable excision efficiency was observed among different independent transformants. One out of ten independent CK2L6-SC retransformants had a 100% *GUS*-negative progeny. As confirmed by molecular analysis, the absence of *GUS* staining was due to *CRE*-mediated excision of the *GUS* gene. In the other nine CK2L6-SC retransformants, the percentage of *GUS*-negative and *GUS* mosaic plants varied, but all transformants displayed *CRE*-mediated recombination. Different excision efficiencies are probably related to different expression levels of the *CRE* recombinase. Indeed, upon transformation, the T-DNA is often randomly integrated into multiple copies, resulting in silencing of the resident T-DNA transgenes and variability of transgene expression (Muskens et al. 2000; Van Houdt et al. 2000; De Buck et al. 2001). Thus, variable *CRE* activity levels could explain the

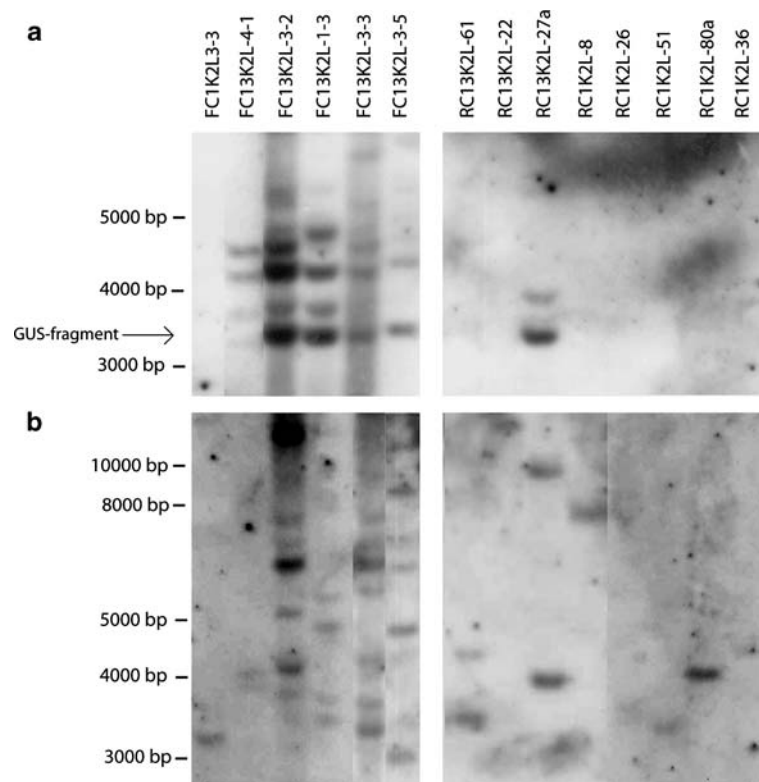


Fig. 5 DNA gel blot analysis on *Hind*III-*Sac*I-digested DNA of FC1K2L, FC13K2L, RC1K2L and RC13K2L retransformants. **(a)** *GUS* excision analysis. A *GUS* fragment of 3199 bp was observed after hybridization with the *GUS* probe when the *GUS* gene is still present in the genome. When *GUS* excision occurred in all cells of the transformant, no *GUS* fragment was detected. The expected *GUS*

fragment is indicated. **(b)** Integration pattern analysis. DNA gel blot analysis with the NPTII probe. On top of each lane, the name is given of the transformant from which DNA was prepared. Left panels, floral dip retransformants; right panels, root tissue retransformants. Seedlings were grown for two weeks on K1 medium supplemented with 50 mg/l kanamycin

complete or partial *CRE*-mediated excision of the *GUS* gene in different transformants. Real-time PCR analysis of transformants CK2L6-SC11 and CK2L6-SC13 demonstrated a correlation between *CRE* mRNA expression levels and observed *CRE* activity. In addition, transformation of the CK2L6 plants with the *CRE* recombinase, controlled either by the constitutive, but weaker, nopaline synthase promoter or the heat-shock-induced promoter *Gm*hsp 17.6L of soybean (*Glycine max*) (Severin and Schöffl 1990) did not result in efficient excision of the *loxP*-flanked *GUS* gene, because *GUS*-negative sectors were observed with a very low frequency (data not shown). This observation suggests that the nopaline synthase and the used heat-inducible promoter were too weak to obtain the required *CRE* recombinase levels.

Subsequently, two *CRE*-expressing plants were retransformed with the *loxP*-containing K2L T-DNA by floral dip and root tissue transformation. A difference with the previous approach is that upon transformation with the K2L T-DNA, multiple copies of the K2L T-DNA can integrate into the *CRE*-expressing plant. In addition, the K2L T-DNA can integrate into different loci in the genome, although mostly multiple T-DNA copies tend to integrate at the same locus (De Block and Debrouwer 1991; De Neve et al. 1997). Different *CRE* recombination rates were observed between the two *CRE*-expressing lines. Quantitative PCR revealed lower *CRE* mRNA levels in Cre13 plants than in Cre1 plants, explaining the less efficient *GUS* excision in the Cre13 than in the Cre1 retransformants. Because both *CRE*-expressing

plants are single-locus transformants derived from transformation with the same *P35S-CRE* gene, the two-fold difference in *CRE* mRNA levels is most probably correlated with the transgene locus structure. Root explant transformation in the two *CRE*-expressing lines resulted in almost all *GUS*-negative T2 progenies. Thus, the *CRE*-mediated excision of the target was clearly better after root tissue transformation than after floral dip transformation. We postulate that this difference in excision rate is due to the fact that fewer T-DNA copies are integrated after root explant transformation than after floral dip transformation. As a consequence, a lower amount of *CRE* proteins would be needed in the RC1K2L and RC13K2L retransformants. Also, this hypothesis could explain that *GUS* excision efficiency is comparable in the CK2L6-SC13 and RC13K2L retransformants with only two allelic and on average one or two K2L T-DNA copies, respectively. The same *CRE* mRNA levels were not sufficient for efficient excision in the FC13K2L retransformants with complex T-DNA integration patterns. Moreover, also the used transformation method to introduce the K2L T-DNA in itself might affect the excision efficiency. Indeed, the expression of the *P35S-CRE* gene and/or the ability of different cell types to respond to *CRE*-mediated recombination can contribute to the differences noted between the two used transformation methods. The *P35S-CRE* expression might be higher in the root explant transformation-responsive cells than in the female gametocyte, or the chromatin might be less susceptible to *CRE* recombination by applying floral dip.

In conclusion, although the efficiency of *CRE*-mediated excision of the *loxP*-flanked *GUS* gene was variable, we obtained *CRE*-expressing *Arabidopsis* transformants in which a *loxP*-flanked *GUS* gene was fully deleted and the deleted locus transmitted to the next generation. In addition, the results demonstrate that a rather high *CRE* expression level is needed when constructs are transformed into the *CRE*-expressing background by floral dip, whereas lower *CRE* mRNA levels are required to obtain the same frequency of fragment excision after root explant transformation. Finally, our study revealed that *CRE*-mediated excision can be maximized by experimentally identifying a highly effective *CRE*-expressing line that can be preferentially used for further transformations with a *loxP*-flanked fragment.

Materials and methods

Plasmid construction for HSC T-DNA, *CRE* T-DNA, and K2L T-DNA

For cloning of pHSC, the *CRE*-coding region was amplified by PCR from the pMM23 plasmid (Dale and Ow 1990) with primers including *SacII* and *NcoI* sites (forward *CRE*-specific primer 5' tccccgcggttgacatgtccaatttactgacc3' and reverse *CRE*-specific primer 5' cat-gccatgggaattcttactaatcgccatcttcc3'). The *NcoI/SacII* *CRE* fragment of 1049 bp was cloned upstream of the 35S terminator, resulting in pCreT35S. Subsequently, the *SacII/ApaI* CreT35S fragment of 1276 bp was cloned into the pPZP200 binary vector (Hajdukiewicz et al. 1994) with the hygromycin selectable marker, resulting in the pHCreT35S plasmid. To create a GATEWAYTM-compatible vector pHGWC, the GW cassette *rfA* (Invitrogen, Carlsbad, CA, USA) for cloning different promoters was cloned upstream of the *CRE* recombinase. The P35S sequence was amplified by PCR with pXD610 DNA (De Loose et al. 1995) as a template. To obtain the P35S-entry clone, the PCR products flanked by attB sites were recombined into the pDONR201-Km^R vector. The T-DNA vector pHSC was generated by an LR reaction in which the P35S entry clone was incubated with the pHGWC destination vector in the presence of LR clonase (Invitrogen).

The plasmid pK2L610, described by De Buck et al. (1998), carries the pK2L610 T-DNA, referred to as the K2L T-DNA throughout the text. For the construction of the *CRE* T-DNA, the reader is referred to De Buck et al. (2000).

Agrobacterium-mediated plant transformation and selection of transformants

The transformation vector pHSC was introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1Rif^R, containing the pMP90 plasmid (Koncz and Schell 1986). The Cre1 and Cre13 lines were obtained after root explant transformation of *Arabidopsis thaliana* (L). Heynh. (ecotype C24) (Valvekens et al. 1988) and selection on phosphinothricin (10 mg/l) and those containing one locus were retained for further analysis. Transformation

of CK2L6 plants (Theuns et al. 2002) with the HSC T-DNA was performed by the floral dip method (Clough and Bent 1998) to obtain transformants into the *lox* target background. Cre1 and Cre13 plants were transformed with the K2L T-DNA (De Buck et al. 1998) by floral dip (Clough and Bent 1998) and by root tissue transformation (Valvekens et al. 1988) to obtain transformants in the CRE background. Seeds of the dipped plants were harvested and sown on K1 medium supplemented with 20 mg/l hygromycin (for HSC T-DNA) or 50 mg/l kanamycin (for K2L T-DNA) for selection of primary transformants. The K1 medium used was germination medium supplemented with 0.5 g/l of *N*-morpholinoethanesulfonic acid. Selected primary transformants (T1) were self-fertilized and T2 seeds were collected.

Plant DNA preparation and DNA gel blot analysis

DNA of *Arabidopsis* seedlings was prepared from 10–30 mg frozen plant tissue with the Puregene™ DNA Purification system (Qiagen, Hilden, Germany). The NPTII and GUS probes (probe 2 and 1, respectively; Fig. 1) were prepared according to De Buck et al. (1999). In each lane of 1% agarose gels, 1 µg *Arabidopsis* DNA was loaded. A nonradioactive method (GeneImages random prime labeling module and GeneImages CDP star detection module; GE-Healthcare, Little Chalfont, UK) was used to label and detect the DNA.

Histochemical β-glucuronidase assay

Two-week-old T2 seedlings were fixed in 90% cold acetone for 30 min with continuous shaking. The seedlings were washed with 0.1 M Na₂HPO₄/NaH₂PO₄ buffer (pH 7), incubated in 0.1 M Na₂HPO₄/NaH₂PO₄ buffer (pH 7) containing 10 mM EDTA, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], 1% dimethylsulfoxide, and 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid overnight at 37°C, afterwards washed with 0.1 M Na₂HPO₄/NaH₂PO₄ buffer (pH 7), destained in 90% ethanol, and stored in 70% ethanol. Photographs were taken with a digital camera (AxioCam HRC; Zeiss, Jena, Germany) connected to a Stemi SV11 microscope (Zeiss).

PCR analysis

Genomic DNA was prepared from pooled leaf material of ten T2 plants per CK2L6-SC retransformants and from eight to ten full T2 seedlings of the Cre1 and Cre13 retransformants from 10–30 mg frozen plant tissue with the Puregene™ DNA Purification system (Qiagen). PCR analysis was performed with 50 ng of DNA and primers specific for the non-excised and excised fragments. The primers used were: p3, 5'tgatcctgttctctgtgtgaaatt3'; p4, 5'ttgaaggagatgcactgatttat3'; p5, 5'att-tgcggccgtttaatagtaaattgtaattgt3'. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min; and a final elongation step at 72°C for 5 min. The efficiency of recombination was tested by PCR analysis with p3 + p4 and p3 + p5. When the CRE recombinase was active and the *P35S-GUS* cassette between the two *loxP* sites was deleted, a fragment of 834 bp was generated with p3 + p4. When no deletion of the *P35S-GUS* cassette in the K2L T-DNA occurred, a fragment of 1200 bp was amplified with p3 + p5.

Real-time PCR analysis

RNA was extracted from pooled 2-week-old *Arabidopsis* seedlings grown on K1 medium with TRIzol® reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. Poly d(T) cDNA was synthesized from 2 µg total RNA with Superscript II reverse transcriptase (Invitrogen). Of the obtained cDNA, 1 µl was used for a PCR with actin primers to check for contamination of residual DNA. The following primers were used: forward primer, 5'ccacctgaaaggaagt3' and reverse primer, 5'aaaacaatgggactaaaacgca3'. CRE expression was quantified on an iCycler real-time PCR detection system (BioRad, Hercules, CA, USA) with the qPCR core kit for SYBR Green I (Eurogentec, Seraing, Belgium). PCRs were carried out in triplicate. Relative expression levels were first normalized to *ACTIN2* expression and then to the respective untransformed controls with the 2^{-ΔΔC_T} method (Livak and Schmittgen 2001). Specific primer pairs were designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA, USA):

At3g18780/*ACTIN2*, 5'gttgactacgagcaggagatgg3' and 5'acaaacgagggctggacaag3'; *CRE*, 5'tttcccgcaaacctgaagatg3' and 5'atccgccataaccagt3'.

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