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Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase

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ABSTRACT

In this study *Agrobacterium tumefaciens* transferred DNA (T-DNA) was targeted to a chromosomally introduced *lox* site in *Arabidopsis thaliana* by employing the Cre recombinase system. To this end, *Arabidopsis* target lines were constructed which harboured an active chimeric promoter–*lox*–*cre* gene stably integrated in the plant genome. A T-DNA vector with a promoterless *lox*–neomycin phosphotransferase (*nptII*) fusion was targeted to this genomic *lox* site with an efficiency of 1.2–2.3% of the number of random events. Cre-catalyzed site-specific recombination resulted in restoration of *nptII* expression by translational fusion of the *lox*–*nptII* sequence in the integration vector with the transcription and translation initiation sequences present at the target site, allowing selective enrichment on medium containing kanamycin. Simultaneously, the coding sequence of the Cre recombinase was disconnected from these same transcription and translation initiation signals by displacement, aimed at preventing the efficient reversible excision reaction. Of the site-specific recombinants, 89% were the result of precise integration. Furthermore, ~50% of these integrants were single copy transformants, based on PCR analysis. *Agrobacterium* T-DNA, which is transferred to plant cells as a single-stranded linear DNA structure, is in principle incompatible with Cre-mediated integration. Nevertheless, the results presented here clearly demonstrate the feasibility of the *Agrobacterium*-mediated transformation system, which is generally used for transformation of plants, to obtain site-specific integration.

INTRODUCTION

In mammalian cells, the introduction of DNA at specific chromosomal loci via homologous recombination has successfully been applied to inactivate or modify specific genes (for a review see 1). In plants, gene targeting can be obtained, although it has not been developed into a powerful technique yet, due to the low frequencies (10^{-3}–10^{-6}) with which transfected DNA recombines with a homologous sequence in the genome (for a review see 2). Integration of transfected DNA into the plant genome usually takes place by a process of non-homologous or illegitimate recombination (3,4). This random integration of transgenes, but also variations in copy number and the configuration of the transgene, which may result in gene silencing (for a review see 5), have been implicated to explain differences in gene expression levels between individual transformants. This variation complicates the use of reporter gene-based analysis of promoters and makes the expression of transgenes less predictable. It would thus be desirable to target any transgene to a specific pre-selected chromosomal site in a single copy fashion to eliminate variation in gene expression levels (6). As the efficiency of gene targeting in plants by homologous recombination is low, site-specific recombination systems, like the bacteriophage P1 Cre/*lox* system, offer a great potential. The family of the integrases, to which the Cre/*lox* system belongs, can catalyze recombination between their respective DNA target sites and, depending on the orientation and position of the recombination sites relative to each other, inversion (7), deletion (8), translocation (9,10) or integration (6,11,12) events can be obtained. The relative simplicity of the system (a recombinase protein Cre and 34 bp *lox* recombination sites) and its efficiency in heterologous organisms, including plants (7–9,12–14), makes it a very useful tool for applications. Prerequisites for precise and stable insertional recombination are circular double-stranded (ds)DNA molecules and controlled expression of the Cre recombinase. The latter is essential due to the reversibility of the recombination reaction, which may lead to excision of integrated DNA sequences in the continuous presence of Cre protein. Successful site-specific integration at a chromosomally introduced *lox* site in plants has been reported previously by using direct gene transfer to tobacco protoplasts (12). However, *Agrobacterium*-mediated transformation is the method of choice for gene transfer to plants, due to its high efficiency and the relatively large number of plant species and tissue types that are susceptible to transformation with *Agrobacterium*. Transferred DNA (T-DNA) is transferred from the bacterium as a linear, single-stranded (ss)DNA molecule (15,16), hence conversion to a circular dsDNA molecule must occur within the plant cell to create a suitable substrate for Cre-mediated insertional recombination. The T-DNA vector used in this study contains two directly oriented *lox* sites, flanking the segment of DNA to be integrated, to allow circularization by Cre recombinase. To control *cre* expression we used insertional inactivation of *cre* by promoter displacement. We report site-specific recombination frequencies of up to 2.3% of random events.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli DH5α was used for cloning, practising standard procedures (17). *Escherichia coli* strains were grown in LC

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medium at 37°C (18) supplemented with 25 mg/l kanamycin (Sigma, St Louis, MO) or 100 mg/l carbenicillin (Duchefa, The Netherlands).

Agrobacterium tumefaciens strain MOG101 (19) was used in plant transformation experiments. Binary vectors (see below) were transferred to MOG101 by electroporation (20). *Agrobacterium* strains were grown at 29°C in LC medium supplemented with 100 mg/l kanamycin, 20 mg/l rifampicin and 250 mg/l spectinomycin (Duchefa).

Plasmid constructs

Binary vector pGPTV-bar (21) was used to construct target vector p35S-lox-cre (Fig. 1A). The *gusA* gene, encoding β-glucuronidase, was removed with *Eco*RI and *Sma*I. The *Hin*dIII site was used to introduce a chimeric 35S–*lox*–*cre* gene. To this end, the *cre* coding region from pUC19CRE (22) was cloned in front of the mannopine synthase (*mas*) transcription terminator region of pCGN7344 (23). Using PCR, the ATG start codon was removed, simultaneously introducing a *Bgl*II site, to facilitate construction of an in-frame fusion with a *lox* site, under transcriptional control of the 35S cauliflower mosaic virus (CaMV) promoter region, with a double enhancer sequence and alfalfa mosaic virus (AMV) 5′-untranslated leader (pMOGEN18; MOGEN International, The Netherlands). In Figure 1B the nucleotide sequence of the *lox–cre* fusion is presented.

Integration vector plox-npt-lox consisted of an in-frame *lox–nptII* fusion (Fig. 1B), lacking an ATG start codon and promoter sequences, as well as a chimeric hygromycin phosphotransferase (*hpt*) gene, cloned in binary vector pSDM14 (24). The *hpt* gene was constructed from pTRA151 (25), pnosHPT (26) and pFWP101 (27). An additional *lox* sequence was cloned near the right T-DNA border in direct orientation with the other *lox* site, allowing Cre-mediated circularization at these *lox* sites (Fig. 1A).

Binary vector p35S-lox-npt was used as a control to assay illegitimate recombination frequencies. The *nptII* open reading frame, devoid of its start codon, and octopine synthase (*ocs*) transcription termination sequence of pSDM56 (28) were cloned in-frame with the 35S–ATG–*lox* fusion and inserted into binary vector pSDM14. This vector can easily be distinguished from recombination events by PCR analysis to exclude the possibility of contamination with this vector in selection plates for recombinants.

An ‘excision binary vector’, containing two *lox* sites in direct orientation separated by the chimeric bialaphos resistance (*bar*) gene from pGPTV-bar (21) with the tumor morphology large gene (*tml*) transcription termination region originating from pTRA151 (25), was constructed. One *lox* site was in-frame with the 35S CaMV promoter with double enhancer sequence, while the other *lox* sequence was fused translationally to the *nptII* coding region. Only upon Cre-mediated excision of the intervening *bar* gene was a functional 35S–*lox–nptII* gene created. This vector was used to assay the *in vivo* activity of Cre recombinase in transgenic target lines (see Results).

Detailed information about plasmid constructs is available on request.

Plant material and growth conditions

Arabidopsis thaliana ecotype C24 and transgenic lines thereof were used. Greenhouse and tissue culture conditions were as described in Vergunst *et al.* (29). Chromosomal DNA was isolated from plants that were grown in potting soil mixed with sand.

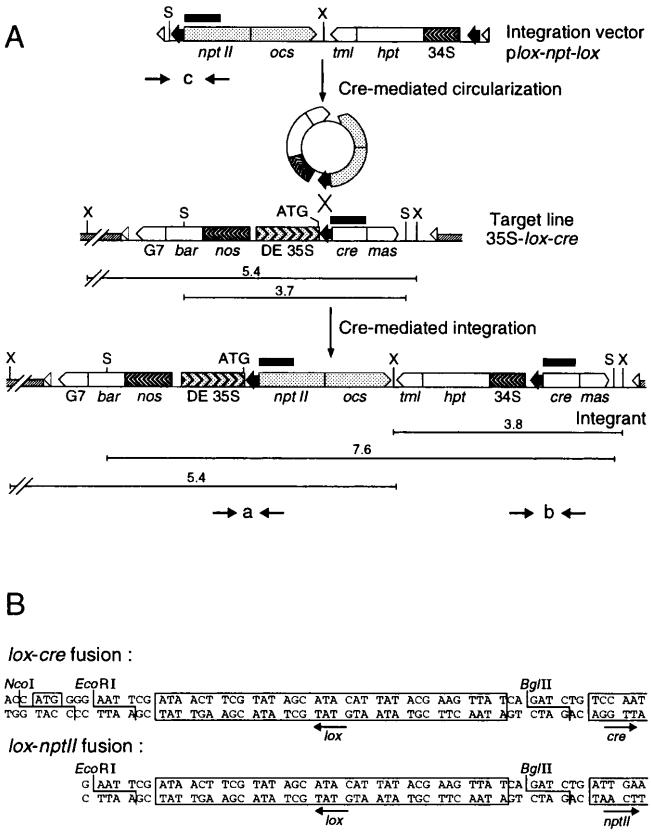

Figure 1. Strategy used to obtain Cre-mediated integration at a chromosomally introduced *lox* site. (**A**) Cre-mediated circularization of a T-DNA vector (plox-npt-lox) at two directly oriented *lox* sites either prior to or after random integration, followed by insertional recombination at a chromosomal target locus (35S–*lox–cre*), stably introduced in *A.thaliana*. X, *Xba*I; S, *Sal*I; small arrows indicate primer binding sites (a, b and c); black boxes, probes; short arrow, *lox* site (the direction of the arrowhead indicates the orientation of the ATG bases present in the 8 bp asymmetric spacer region). Open triangle, *A.tumefaciens* left and right border repeat; >>>>, promoter regions. (**B**) Nucleotide sequence of the 35S–*lox*–cre fusion in the target line and the *lox–nptII* fusion in the integration vector. Restoration of *nptII* expression by site-specific integration simultaneously leads to displacement of *cre* from transcription and translation initiation signals.

Plant transformation

Agrobacterium-mediated transformation of *Arabidopsis* root explants (29) was adapted from Valvekens *et al.* (30). Root explants of wild-type *Arabidopsis* were co-cultivated with *A.tumefaciens* strain MOG101(p35S-lox-cre), in order to isolate target plants. Shoot-inducing medium (SIM) containing 20 mg/l phosphinothricin (PPT), 100 mg/l vancomycin and 500 mg/l carbenicillin (Duchefa) was used for selection of transformants (T1). One single copy transformant was selected on the basis of genetic and Southern blot analysis (data not shown). T2 or T3 seeds from this target line were sown in medium supplemented with PPT (5 mg/l). The resistant seedlings, which were either hemizygous or homozygous for the target sequence (2:1), were used in recombination experiments. Root explants excised from the seedlings were co-cultivated with *Agrobacterium* strain MOG101(plox-npt-lox) followed by selection for recombinants on SIM with 50 mg/l kanamycin (Sigma) and 100 mg/l timentin

(Duchefa). Random integration frequencies were estimated by co-cultivation of target roots with control strain MOG101(p35S-lox-npt). To determine the number of kanamycin resistant (Km^r) background calli due to translational fusion with endogenous plant genes, wild-type C24 roots were co-cultivated with MOG101(plox-npt-lox). The number of Km^r calli per root explant was determined 3–5 weeks after co-cultivation.

DNA isolation and Southern analysis

Two grams of leaf material of ~20 T2 plants, just prior to bolting, was collected and frozen in liquid nitrogen and stored at –80°C. After isolation (31), 2 µg chromosomal DNA were digested with appropriate restriction enzymes and separated on a 0.7% TAE agarose gel, including 20 ng digoxigenin-labeled marker DNA II (Boehringer Mannheim, Germany). DNA blotting was performed on Boehringer membrane according to the manufacturer's recommendations. Incorporation of digoxigenin-dUTP in the probes was performed according to Lahaye *et al.* (32). The non-radioactive chemiluminescent method for detection of DNA (Boehringer; 33) was applied, using the substrate CDP-star™.

PCR analysis

Isolation of chromosomal DNA from callus or leaf material was performed essentially as described by Lassner *et al.* (34). PCR reactions were performed in a Perkin-Elmer Thermocycler 480 with primers: a1, 5′-GAACTCGCCGTAAAGACTGGCG-3′; a2, 5′-GCGCTGACAGCCGGAACACG-3′; b1, 5′-GGTAATC-TTTTTTGTACACTTGCTCG-3′; b2, 5′-CCAGCAGGCGCA-CCATTGC-3′; c1, 5′-CGAAGTCGACGGATCCGGTACCG-3′; c2, 5′-CACCATGATATTCGGCAAGC-3′ (Fig. 1A). A standard protocol of 30 cycles was used: 1 min 95°C, 2 min 57°C and 2 min 72°C. The reaction mixture (50 µl) contained 50 ng template DNA, 165 ng of each primer, 200 µM dNTPs and 0.2 U SuperTaq polymerase (HT Biotechnology Ltd, Cambridge, UK).

RESULTS

Experimental design

Transgenic *Arabidopsis* plants with a *lox* target sequence (35S–*lox–cre*) stably integrated in the genome and expressing *cre* (see below) were transformed with a T-DNA vector carrying a promoterless *lox–nptII* fusion, which in addition lacks the ATG start codon (Fig. 1A). Preferential selection of Cre-mediated site-specific recombination of the T-DNA vector at the genomic *lox* site, but not random integration, was based on restoration of *nptII* expression by translational fusion to the 35S promoter present at the target locus. Control of *cre* expression is an essential step in stabilization of recombination events, because presence of Cre enzyme after site-specific integration will lead to subsequent loss of the once integrated vector. In this study, the integration reaction itself would lead to inactivation of *cre* expression, due to loss of the promoter region and ATG start codon, which are provided to the *lox–nptII* fusion instead. The nucleotide sequences of these fusions are presented in Figure 1B.

Selection of target plants expressing Cre

Arabidopsis plants containing a single copy of the target sequence (see Materials and Methods) were tested for *in vivo* activity of the chimeric Cre protein. To this end, root explants were transformed with an excision vector (see Materials and Methods) which harboured a silent *nptII* gene, due to separation of the coding sequence from its transcription and translation initiation signals by two directly oriented *lox* sites flanking a *bar* gene. Transformation of the vector to wild-type *Arabidopsis* roots yielded no Km^r calli, as expected. Transformation of the vector to roots excised from target plants, however, yielded Km^r calli, which indicated that Cre/*lox*-mediated excision of the *bar* gene and restoration of *nptII* expression had occurred. This confirmed expression of the *cre* gene present in the genome of the target line, which was further used in site-specific recombination experiments.

Transformation of target plants and selection of site-specific recombinants

Agrobacterium-mediated transformation of root explants excised from the target line with strain MOG101(plox-npt-lox) was followed by selection of site-specific recombinants on medium containing kanamycin. For estimating the random integration frequency control strain MOG101(p35S-lox-npt), with a T-DNA vector carrying an active *nptII* gene, was used in parallel. Two independent experiments, summarized in Table 1, yielded a total of 44 Km^r calli after transformation of target roots (35S–*lox–cre*) with integration vector plox-npt-lox. These calli were selected with a frequency of 2.5×10^{-2} and 2.3×10^{-2} calli/root explant, whereas random integration with the control vector yielded 2 and 1 calli/root explant respectively. By comparison of both frequencies the ratio of site-specific recombinants to random integrants was calculated to be 1 in 81 and 1 in 43. Regeneration occurred in 85 and 87% of the Km^r calli, while 69 and 50% respectively yielded fertile offspring (Table 1).

Transformation of wild-type C24 with MOG101(plox-npt-lox) in parallel generated a few Km^r calli, probably due to translational fusion of the *lox–nptII* fusion with an endogenous plant gene.

Table 1. Efficiency of site-specific recombination after transformation of target line 35S–*lox–cre* with integration vector plox-npt-lox

Experiment	Random integration frequency[a]	No. of explants	No. of Km^r calli	Absolute recombination frequency[b]	Ratio[c]	Regeneration efficiency (%)[d]	Seed set (%)
1	2	1050	26	2.5×10^{-2}	1:81	85	69
2	1	780	18	2.3×10^{-2}	1:43	87	50

[a]Random integration frequency of p35S-lox-npt control vector: number of Km^r calli per root explant.
[b]Absolute recombination frequency: number of Km^r calli per root explant.
[c]Ratio of number of Km^r calli after transfomation of 35S–*lox–cre* with plox-npt-lox (b) per number of random events (a).
[d]Percentage of calli regenerating shoots.

Site-specific integration of plox-npt-lox at a chromosomal 35S–*lox*–*cre* target

PCR analysis was performed on DNA samples from the 44 Km^r calli isolated in the recombination experiments as well as on five Km^r background calli derived after transformation of the integration vector to wild-type *Arabidopsis*. Primer sets a and b (Fig. 1A) will lead to the amplification of diagnostic fragments of 0.65 kb (indicative of a precise left junction) and 1.0 kb (indicative of a precise right junction) respectively in DNA preparations from precise site-specific integrants. These primer sets will not lead to amplification of specific fragments in DNA from random integrants or target plants. In this way we found that 39 (89%) Km^r calli indeed contained both recombinant junctions and thus that kanamycin resistance in these lines likely resulted from precise site-specific integration of the integration vector (data not shown). In DNA samples from the other resistant calli a 0.65 kb fragment was amplified using primer set a, indicating recombination with the 35S–*lox* target, but primer set b did not lead to amplification of the expected 1.0 kb fragment. This is indicative of rearrangements or deletion events at the target locus. Primer combinations a and b did not result in amplification of specific fragments in DNA isolated from the five calli originating from transformation of wild-type plants or target plants, as expected.

Next, we determined whether the recombinants with a targeted integration of T-DNA contained other, randomly integrated copies of the T-DNA. To this end primer combination c (Fig. 1A) was used. Random integration of vector plox-npt-lox would allow detection of a 0.7 kb fragment, whereas precise integration would lead to loss of the left primer binding site. About 50% of the integrants did amplify a specific fragment. This was also confirmed for a number of these lines by Southern blot analysis. Due to the position of the left primer binding site, close to the left border repeat, border truncation events might have remained undetected, which indicates that the actual proportion of targeted single copy integrants could be somewhat lower.

For Southern blot analysis chromosomal DNA from target plants (T) and three individual recombinants was subjected to digestion with *Sal*I and *Xba*I (Figs 1A and 2). Two identical blots were prepared and hybridized with a *nptII* (Fig. 2, right panel) and *cre* (Fig. 2, left panel) probe respectively. Hybridization of 7.6 *Sal*I and 5.4 kb *Xba*I fragments occurred with a *nptII* probe in the recombinants, whereas chromosomal DNA of target plants did not hybridize. This is consistent with precise site-specific integration and confirmed results obtained with PCR. In recombinant 2, additional hybridizing fragments with a *nptII* probe are indicative of random integration events, besides the site-specific integration event, which confirmed results obtained by PCR for this site-specific integrant. The appearance of new 7.6 (*Sal*I) and 3.8 kb (*Xba*I) fragments respectively in the recombinants after hybridization with a *cre* probe are also diagnostic for precise site-specific integration. Instead, 3.7 kb *Sal*I and 5.4 kb *Xba*I fragments were present in the original target plant. The occurrence of these target fragments also in the site-specific integrants prompted us to further analyse the origin of these bands (see below).

Instability of the site-specific integrants

The transformation experiments were performed on plant cells that were either homozygous or hemizygous for the target locus. Site-specific integrants might thus be derived from cells

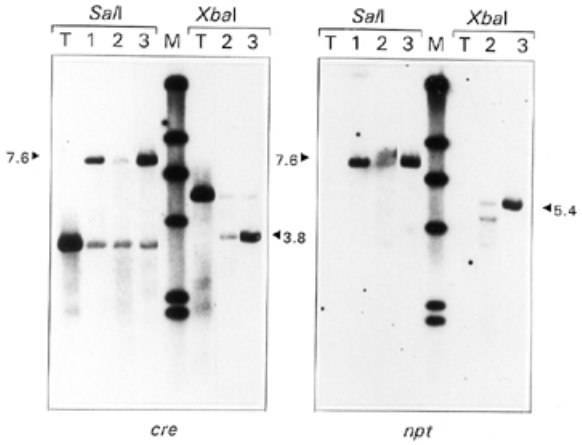

Figure 2. Southern blot analysis of chromosomal DNA from target plant (T) and three individual site-specific integrants (1–3) digested with *Sal*I and *Xba*I and hybridized with *cre* (left panel) or *nptII* (right panel) probes respectively. Indicated (triangles) are expected recombinant fragments (in kb) as shown in Figure 1A. DNA from recombinant 1 was only partially digested with *Xba*I and therefore not included.

homozygous for the target locus, in which site-specific integration of the T-DNA occurred at only one of the two target *lox* sites. This would explain the 3.7 kb *Sal*I and 5.4 kb *Xba*I target fragments seen in the Southern blot analysis of the site-specific integrants (Fig. 2, left panel), besides the expected 7.6 and 3.8 kb fragments. Such plants would be homozygous for the *bar* gene, but hemizygous for the *nptII* gene. Alternatively, if the site-specific integrants were derived from cells hemizygous for the target locus, they would segregate on PPT in the T2 generation. If the integrants showing target sequences on the Southern blot segregated for the *bar* gene this would be an indication of instability of the recombined locus, regenerating the original target locus in some of the cells. We therefore analyzed for segregation of the *bar* gene in the T2 of 16 site-specific integrants. All except for one integrant (including integrants 1–3 in Fig. 2) showed segregation (χ^2 test for goodness of fit, $P > 0.05$) for PPT resistance. Further genetic analysis of 19 individual integrants on kanamycin confirmed some instability in the T2 generation. Besides segregating resistant (indicating stable incorporation) and sensitive seedlings, some seedlings became sensitive to kanamycin during plant development, seen as a bleaching of the leaves. A few site-specific integrants yielded mostly sensitive offspring.

DISCUSSION

Here, we describe that *Agrobacterium* T-DNA can be integrated precisely at a predetermined position in the *Arabidopsis* genome using the Cre/*lox* recombination system. A chromosomally introduced *lox* site was targeted with a frequency of 1.2–2.3%. Eighty nine percent of the recombinants resulted from precise site-specific integration. Controlling the reversibility of the recombination reaction by insertional inactivation of *cre* most likely aided in isolation of these recombinants.

It can be concluded from PCR and Southern data that all Km^r calli detected after transformation of the target line with the integration vector were isolated due to activation of the *nptII* gene

by site-specific recombination. The background calli isolated after transformation of wild-type plants with the integration vector indicated random fusion with endogenous plant genes. Background calli, solely due to activation of the *nptII* gene by random fusion to endogenous genes, were not detected in the actual recombination experiments. The absence of this second class of Km^r calli in recombination experiments can be explained by the fact that a randomly integrated vector is prone to excision at the two *lox* sites by Cre enzyme, present in the target cells. Excision of random events may be prevented in cells in which simultaneous insertional, so targeted, integration of the T-DNA occurred, leading to inactivation of the *cre* gene.

Agrobacterium is widely used for transformation of plants, due to the high efficiency of transfer and relatively large number of plant species and tissue types that are competent for transformation. A broad application of site-specific integration in plants, therefore, requires that site-specific integrants can be obtained after delivery of T-DNA by *Agrobacterium*. Albert and co-workers (12) demonstrated Cre/*lox*-mediated site-specific integration of plasmid DNA after direct gene transfer to tobacco protoplasts. The relevance of the results described here is that we could demonstrate that the single-stranded linear nature of the T-DNA (15,35) forms in principle no barrier to obtaining precise site-specific integration, which requires circular dsDNA molecules. In fact, frequencies of site-specific integration in the range of frequencies found in mammalian systems (36) could be obtained. The fate of T-DNA molecules within the plant cell nucleus and the mechanism leading to illegitimate integration of T-DNA still remains largely unknown. It is unclear whether T-DNA integration requires ds or ssDNA, although extrachromosomal second strand synthesis can occur, as shown by transient expression studies (37) and extrachromosomal recombination experiments (38,39). A dsDNA structure is essential for Cre-mediated recombination. We used a T-DNA vector with two directly oriented *lox* sites. Upon conversion to a dsDNA substrate, Cre recombinase can mediate circularization. The circular dsDNA molecule may then be integrated site-specifically at the target *lox* sequence. The conversion to a dsDNA substrate may be a rate limiting step for Cre-mediated extrachromosomal circularization and would allow the T-DNA to participate in a process of illegitimate recombination with genomic DNA. Subsequent Cre-mediated excision would then result in footprints at the initial random integration position. We are currently testing whether such footprints are present in site-specific integrants by addition of a reporter gene outside the region of the T-DNA to be targeted. Extrachromosomal circularization results in the formation of a *lox* circle and a residual small linear T-DNA with a *lox* sequence, which might integrate randomly as well. Preliminary data indicate that indeed footprints and/or random events do occur frequently. These do not need to represent a problem, however, as the addition of negative selectable markers outside the region to be targeted would allow isolation of site-specific integrants without additional random events or footprints by breeding.

Using a displacement strategy to control *cre* expression, combined with mutant *lox* sites, Albert *et al.* (12) showed site-specific integration frequencies similar to random integration after direct gene transfer to tobacco protoplasts. The use of circular dsDNA molecules, together with the use of mutant *lox* sites, may have resulted in these high frequencies. Wild-type *lox* sites, in their experiments, did not result in any resistant calli, indicating the positive effect of mutant *lox* sites on stability. The fact that we did obtain site-specific integrants in a similar displacement strategy, using wild-type *lox* sites, suggests that the loss of both transcription and translation initiation signals after site-specific integration may have aided in preventing *cre* expression and thus excision. The partial instability of the recombinants in the T2 generation, as evidenced by hybridizing fragments of the target sequence (Fig. 2) and partial sensitivity to kanamycin, indicates a very low level of *cre* expression that can drive the efficient excision reaction. We found that mainly hemizygous cells, which formed 66% of the starting population used for transformation, had survived selection for recombination on kanamycin. Apparently, in cells homozygous for the target allele, both expressing *cre*, stable integration at one of the *lox* sites was prevented by expression of *cre* from the second allele, leading to rapid reversion of integration events. As there was no second target allele expressing *cre* present in the site-specific integrants, the method of stabilizing the recombination event by Cre displacement seems incompletely tight and leakage of *cre* expression, even after displacement of transcription and translation initiation signals, occurs from the recombinant locus. The incorporation of mutant *lox* sites in our system may aid in stabilizing the targeted T-DNA molecules.

Recently, Bethke and Sauer (40) published the use of a replacement strategy using a wild-type and a heterospecific *lox* site, both in the genome and in the vector, to allow a Cre-catalyzed double reciprocal crossover in mammalian cells. This strategy, also known as exchange (41), led to highly efficient targeted integration in these cells. This approach seems very useful for T-DNA-mediated site-specific recombination and may lead to development of a more efficient system, applicable to many plant species. Nevertheless, our current results already indicate that the Cre/*lox* system can be used to target *Agrobacterium* T-DNA with a reasonable efficiency to a specific site in the plant genome.

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REFERENCES

1 Brandon,E.P., Idzerda,R.L. and McKnight,G.S. (1995) *Curr. Biol.*, **5**, 625–634.
2 Morton,R. and Hooykaas,P.J.J. (1995) *Mol. Breeding*, **1**, 123–132.
3 Mayerhofer,R., Koncz-Kalman,Z., Nawrath,C., Bakkeren,G., Crameri,A., Angelis,K., Redei,G.P., Schell,J., Hohn,B. and Koncz,C. (1991) *EMBO J.*, **10**, 697–704.
4 Gheysen,G., Villarroel,R. and Van Montagu,M. (1991) *Genes Dev.*, **5**, 287–297.
5 Maessen,G.D.F. (1997) *Acta Bot. Neerland.*, **46**, 3–24.
6 Fukushige,S. and Sauer,B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7905–7909.
7 Medberry,S.L., Dale,E., Qin,M. and Ow,D.W. (1995) *Nucleic Acids Res.*, **23**, 485–490.
8 Dale,E.C. and Ow,D.W. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10558–10562.
9 Qin,M., Bayley,C., Stockton,T. and Ow,D.W. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 1706–1710.
10 Smith,A.J.H., De Sousa,M.A., Kwabi-Addo,B., Heppell-Parton,A., Impey,H. and Rabbitts,P. (1995) *Nature Genet.*, **9**, 376–385.

11 Baubonis,W. and Sauer,B. (1993) *Nucleic Acids Res*., **21**, 2025–2029.
12 Albert,H., Dale,E.C., Lee,E. and Ow,D.W. (1995) *Plant J*., **7**, 649–659.
13 Odell,J., Caimi,P., Sauer,B. and Russell,S. (1990) *Mol. Gen. Genet*., **223**, 369–378.
14 Odell,J.T., Hoopes,J.L. and Vermerris,W. (1994) *Plant Physiol*., **106**, 447–458.
15 Tinland,B., Hohn,B. and Puchta,H. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 8000–8004.
16 Zupan,J. and Zambryski,P. (1997) *Crit. Rev. Plant Sci*., **16**, 279–295.
17 Sambrook,J., Fritsch,E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.
18 Hooykaas,P.J.J., Klapwijk,P.M., Nuti,M.P., Schilperoort,R.A. and Rörsch,A. (1977) *J. Gen. Microbiol*., **98**, 477–484.
19 Hood,E.E., Gelvin,S.B., Melchers,L.S. and Hoekema,A. (1993) *Transgenic Res*., **2**, 208–218.
20 Mattanovich,D., Rüker,F., Da Câmara Machado,A., Laimer,M., Regner,F., Steinkellner,H., Himmler,G. and Katinger,H. (1989) *Nucleic Acids Res*., **17**, 6747.
21 Becker,D., Kemper,E., Schell,J. and Masterson,R. (1992) *Plant Mol. Biol*., **20**, 1195–1197.
22 Mozo,T. and Hooykaas,P.J.J. (1992) *Mol. Gen. Genet*., **236**, 1–7.
23 Comai,L., Moran,P. and Maslyar,D. (1990) *Plant Mol. Biol*., **15**, 373–381.
24 Offringa,R. (1992) PhD thesis, Leiden University, The Netherlands.
25 Zheng,Z., Hayashimoto,A., Li,Z. and Murai,N. (1991) *Plant Physiol*., **97**, 832–835.
26 Halfter,U., Morris,P. and Willmitzer,L. (1992) *Mol. Gen. Genet*., **231**, 186–193.
27 Sanger,M., Daubert,S. and Goodman,R.M. (1990) *Plant Mol. Biol*., **14**, 433–443.
28 De Groot, M.J.A. (1992) PhD thesis, Leiden University, The Netherlands.
29 Vergunst,A.C., De Waal,E.C. and Hooykaas,P.J.J. (1998) In Martinez-Zapater,J. and Salinas,J. (eds), *Methods in Molecular Biology*, Vol. 82, *Arabidopsis Protocols*. Humana Press, Totowa, NJ, pp. 227–244.
30 Valvekens,D., Van Montagu,M. and Van Lijsebettens,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5536–5540.
31 Bernatzky,R. and Tanksley,S.D. (1986) *Theor. Appl. Genet*., **72**, 314–321.
32 Lahaye,T., Rueger,B., Toepsch,S., Thalhammer,J. and Schulze-Lefert,P. (1996) *BioTechniques*, **21**, 1067–1072.
33 Neuhaus-Url,G. and Neuhas,G. (1993) *Transgenic Res*., **2**, 115–120.
34 Lassner,M.W., Peterson,P. and Yoder,J.I. (1989) *Plant Mol. Biol. Rep*., **7**, 116–128.
35 Yusibov,V.M., Steck,T.R., Gupta,V. and Gelvin,S.B. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2994–2998.
36 Sauer,B. (1994) *Curr. Opin. Biotechnol*., **5**, 521–527.
37 Mozo,T. and Hooykaas,P.J.J. (1992) *Plant Mol. Biol*., **19**, 1019–1030.
38 Offringa,R., de Groot,M.J.A., Haagsman,H.J., Does,M.P., van den Elzen,P.J.M. and Hooykaas,P.J.J. (1990) *EMBO J*., **9**, 3077–3084.
39 Bilang,R., Peterhans,A., Bogucki,A. and Paszkowski,J. (1992) *Mol. Cell. Biol*., **12**, 329–336.
40 Bethke,B. and Sauer,B. (1997) *Nucleic Acids Res*., **25**, 2828–2834.
41 Schlake,T. and Bode,J. (1994) *Biochemistry*, **33**, 12746–12751.