

Stable Recombinase-Mediated Cassette Exchange in *Arabidopsis* Using *Agrobacterium tumefaciens*¹

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Site-specific integration is an attractive method for the improvement of current transformation technologies aimed at the production of stable transgenic plants. Here, we present a Cre-based targeting strategy in *Arabidopsis* (*Arabidopsis thaliana*) using recombinase-mediated cassette exchange (RMCE) of transferred DNA (T-DNA) delivered by *Agrobacterium tumefaciens*. The rationale for effective RMCE is the precise exchange of a genomic and a replacement cassette both flanked by two heterospecific *lox* sites that are incompatible with each other to prevent unwanted cassette deletion. We designed a strategy in which the coding region of a *loxP/lox5171*-flanked bialaphos resistance (*bar*) gene is exchanged for a *loxP/lox5171*-flanked T-DNA replacement cassette containing the neomycin phosphotransferase (*nptII*) coding region via *loxP/loxP* and *lox5171/lox5171* directed recombination. The *bar* gene is driven by the strong 35S promoter, which is located outside the target cassette. This placement ensures preferential selection of RMCE events and not random integration events by expression of *nptII* from this same promoter. Using root transformation, during which Cre was provided on a cotransformed T-DNA, 50 kanamycin-resistant calli were selected. Forty-four percent contained a correctly exchanged cassette based on PCR analysis, indicating the stringency of the selection system. This was confirmed for the offspring of five analyzed events by Southern-blot analysis. In four of the five analyzed RMCE events, there were no additional T-DNA insertions or they easily segregated, resulting in high-efficiency single-copy RMCE events. Our approach enables simple and efficient selection of targeting events using the advantages of *Agrobacterium*-mediated transformation.

Plant transformation is a fundamental technique in plant science research as well as in the production of transgenic crops. Independent of the transformation method that is applied, gene silencing and variable transgene expression are a major problem for the production of stable transgenic plants. Transgene instability and variation in expression levels are mostly caused by transgene integration patterns (multiple insertions often organized as complex loci or rearranged transgene inserts) and genomic location (Peach and Velten, 1991; Kohli et al., 2003; Francis and Spiker, 2005). In addition, transgenes become integrated at random positions in the plant genome (Kim et al., 2007), which may result in unwanted mutations due to insertion in active genes. Site-specific recombination systems have been utilized for the directed and precise

single-copy integration at predetermined genomic positions to circumvent the problems mentioned above.

The Cre/*lox* system of bacteriophage P1 has been developed as a versatile tool due to its simplicity and activity in heterologous systems (Ow, 2002; Gilbertson, 2003). It requires a single recombinase protein, Cre, which does not need additional cofactors to mediate reciprocal recombination between two of its 34-bp *loxP* DNA recognition sites. Cre monomers bind to *loxP* sites at two 13-bp inverted repeats that are separated by an 8-bp spacer region, which gives directionality to the target site. DNA flanked by *loxP* sites positioned in direct orientation will be excised, whereas *loxP* sites in inverted orientation will result in inversion of the *loxP*-flanked DNA. The Cre/*lox* system has been successfully used in plants to remove selectable marker genes that have served their purpose in transformation (Gleave et al., 1999; Corneille et al., 2001; Zuo et al., 2001; Kopertekh et al., 2004) or to resolve complex integrated loci (Srivastava et al., 1999; Srivastava and Ow, 2001, 2002; Zuo et al., 2001). In addition, site-directed integration of DNA molecules at a predetermined site in the genome has been successful in plants, using direct DNA transfer to tobacco (*Nicotiana tabacum*) protoplasts (Albert et al., 1995; Day et al., 2000), DNA delivered via particle bombardment of rice (*Oryza sativa*; Srivastava and Ow, 2002; Srivastava et al., 2004), as well as after *Agrobacterium*-mediated T-DNA delivery to *Arabidopsis* (*Arabidopsis thaliana*) and tobacco (Vergunst and Hooykaas, 1998; Vergunst et al., 1998b; Nanto et al., 2005; Nanto and Ebinuma, 2007).

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The use of site-specific recombination systems for directed integration requires a two-step procedure. First, a target plant line is produced containing a recombination site, which will subsequently be used as a landing platform for integration of transgenes of interest delivered in a second round of transformation. Several strategies have been employed with the aim of obtaining single-copy, stable, site-specific integrants. Target plants with a single genomic *lox* target site have been used successfully for the selection of specific integrants. The reversibility of a Cre-mediated integration reaction required methods to control *cre* expression. These methods include transient expression of Cre (Albert et al., 1995; Vergunst and Hooykaas, 1998) and use of a *cre* promoter-displacement strategy (Albert et al., 1995; Vergunst et al., 1998b), which can be combined with the use of specific mutant *lox* sites (the recombination products of such *lox* sites are no longer able to recombine with each other) to achieve stabilization of the integrated DNA (Albert et al., 1995; Srivastava et al., 2004).

In mammalian systems, efficient recombinase-mediated cassette exchange (RMCE) strategies were developed to overcome the problem of reversible excision. RMCE allows the replacement of a genomic cassette with any desired transgene or DNA construction via a double recombinase-mediated reaction based on the presence of two recombination sites flanking both the genomic and exchange cassettes (Schlake and Bode, 1994; Bouhassira et al., 1997; Baer and Bode, 2001; Lauth et al., 2002; Wallace et al., 2007). A prerequisite for efficient RMCE is that the recombination sites flanking the cassette are not compatible. Otherwise, depending on the orientation of the sites, the cassette might become inverted or deleted from its insertion site in the presence of its cognate recombinase.

For transformation of many plant species, *Agrobacterium tumefaciens* is the preferred method due to its efficiency and simplicity. This warrants the development of an efficient site-specific integration strategy based on T-DNA, but several caveats have to be addressed. In the bacterium, a part of the large tumor-inducing plasmid, called the T-region, is cleaved at the border sequences by the VirD2 protein, and a single-stranded DNA (ssDNA) copy of the bottom strand (T-strand) is released. VirD2 remains covalently attached to the 5'-end of the T-strand. The T-DNA/VirD2 complex and several other Vir proteins are transported via the bacterial type IV secretion system into host cells. In the plant cell, the ssDNA-binding protein VirE2 binds cooperatively to the T-strand and thereby protects it from degradation (Rossi et al., 1996). Both VirD2 and VirE2 contain nuclear localization signals that facilitate transport of the T-complex into the nucleus (Zupan et al., 1996; Citovsky et al., 1997; Ziemienowicz et al., 2001). *Agrobacterium* thus delivers T-DNA as a linear ssDNA molecule (Tinland et al., 1994), which is in principle incompatible with site-specific recombination that requires double-stranded DNA. In addition, for an integration event

at a single chromosomally introduced recombination site, the DNA molecule needs to be circular to prevent chromosome breakage. Based on several studies, it is known that T-DNA becomes double stranded prior to integration (Offringa et al., 1990; Mozo and Hooykaas, 1992; Narasimhulu et al., 1996; Chilton and Que, 2003; Tzvira et al., 2003). Although circle formation of T-DNA by ligation of the two T-DNA borders has been reported (Bakkeren et al., 1989; Bundock et al., 1995; Zhao et al., 2003), the frequency of occurrence remains unclear. Pansegrau and coworkers (1993) have shown that VirD2 can mediate ligation of the two T-DNA borders in vitro. In vivo, circular T-DNA was only recovered when it was adapted with a replication origin. This was the case when a viral replicon was placed on the T-DNA in maize (*Zea mays*) cells (Zhao et al., 2003) and turnip (*Brassica napus*; Bakkeren et al., 1989), or in the presence of the replicator from the yeast (*Saccharomyces cerevisiae*) 2 μ plasmid (Bundock et al., 1995). These data suggest that border ligation may occur, but is not efficient. Previously, in our laboratory, we demonstrated that a Cre/*lox* integration strategy of T-DNA is feasible using a single *loxP* site at the target locus. The placement of two *loxP* sites in the T-DNA replacement vector, allowing Cre-mediated circularization upon entry in the host cell prior to targeting, resulted in the isolation of integrants at workable efficiency (Vergunst et al., 1998b). However, despite the use of methods to control expression of Cre, the integrants were still partially unstable due to the occurrence of the reversible deletion event. In this study, we improve on previously reported *Agrobacterium*-mediated site-specific integration methods by combining RMCE, heterospecific *lox* sites, and Cre delivery via a cotransfer method. For Cre technology, many mutant *lox* sites have been developed. Lee and Saito (1998) demonstrated in an in vitro assay that spacer mutation sites *lox5171* recombined with themselves but not with a wild-type *loxP* site. We designed target and exchange cassettes flanked by *loxP* and *lox5171* in inverted orientation and obtained stable single-copy exchange events in Arabidopsis.

RESULTS

Experimental Design

The rationale for our RMCE strategy is depicted in Figure 1. In a first step, the target construct was introduced in the plant genome by *Agrobacterium*-mediated transformation. This construct contains the bialaphos resistance (*bar*) coding region, flanked by *loxP* and the mutant *lox5171* site in an inverted orientation (Fig. 1A). The *loxP-bar* coding region (Fig. 1E) is expressed from the strong cauliflower mosaic virus (CaMV) 35S promoter adjacent to the *lox*-flanked exchange cassette, providing resistance to phosphinothricin (PPT). The replacement T-DNA contains an exchange cassette with a promoterless neomycin phos-

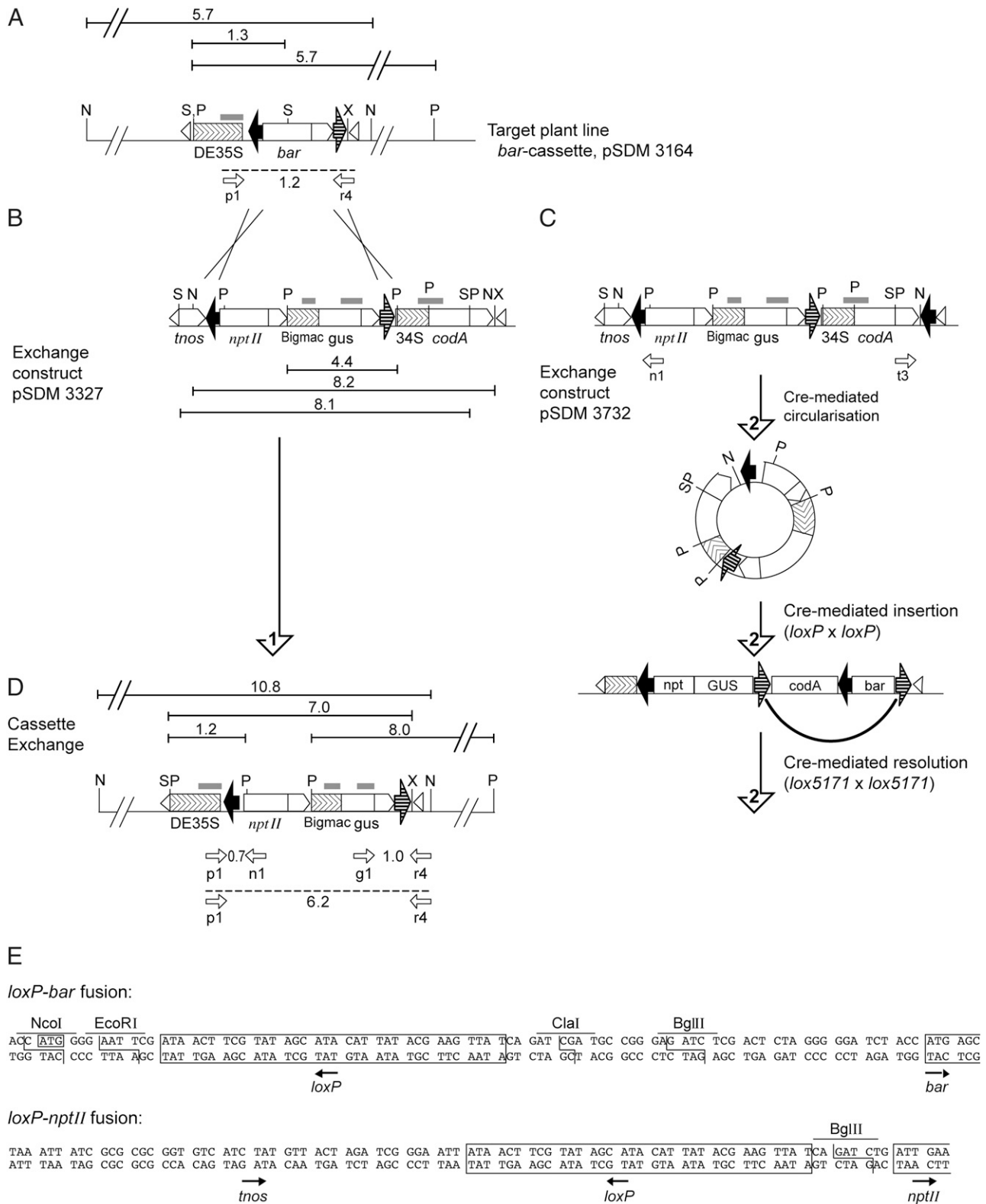


Figure 1. Strategy to obtain Cre-mediated cassette exchange. A, Chromosomally introduced target cassette (T-DNA pSDM3164). The *bar* gene is flanked by the *loxP* site (large black arrow) and the *lox5171* site (large dashed arrow) in inverted orientation, with the strong CaMV 35S promoter and double enhancer sequence (DE35S) adjacent to the cassette providing resistance to PPT. B, Exchange T-DNA pSDM3327 contains the *nptII* coding sequence and *gus* expression unit driven by the BigMac promoter, flanked by *lox* sites in similar configuration as the *bar* cassette. The *nos* transcription terminator, *tnos*, and three translation stop

phototransferase (*nptII*) gene and a β -glucuronidase (*gus*) gene allowing visualization and analysis of gene expression levels. Upon RMCE, the *bar* gene will be replaced by the *nptII* gene, effectively exchanging *bar* gene expression for *nptII* gene expression and allowing preferential selection for recombinants on medium containing kanamycin. Outside the exchange cassette, near the right T-DNA border repeat, the conditional negative selectable marker *codA* is inserted to allow selection of RMCE events in the absence of random insertions expressing *codA*.

We tested two versions of the exchange T-DNA. Plasmid pSDM3327 contains a T-DNA with a simple RMCE cassette (Fig. 1B) flanked by a *loxP* and a *lox5171* site. Using this T-DNA vector, RMCE will be the result of simultaneous *loxP/loxP* and *lox5171/lox5171* recombination between the target locus and exchange T-DNA. We anticipated that simultaneous recombination would be an infrequent event, resulting in chromosome breakage or translocation events, respectively. Therefore, we inserted an additional *loxP* site in direct orientation with the *loxP* site near the right-border repeat, resulting in plasmid pSDM3732 (Fig. 1C). This would allow efficient Cre-mediated circularization of the T-DNA, providing a circular *lox* substrate that may first integrate at the target site by a single *loxP/loxP* or *lox5171/lox5171* recombination step (one of two possible intermediates is depicted in Fig. 1C). In a second step, Cre may resolve the integrated product, resulting in a precise selectable exchange (Fig. 1D). In the case of T-DNA with the simple RMCE cassette, T-DNA border ligation may result in a similar circular substrate, but probably this does not occur frequently (Bundock et al., 1995; Vergunst and Hooykaas, 1998; Zhao et al., 2003). Here, we used a cotransformation approach to introduce Cre recombinase in the same cell as the exchange T-DNAs by means of an *Agrobacterium* strain harboring a T-DNA vector containing a strong expression unit for the *cre* gene.

Target Plant Line

Arabidopsis ecotype C24 root explants were cocultivated with LBA1100 (pSDM3164). Several target lines were selected and the transgenic plants were screened for the presence of single-copy nontruncated inserts using the outermost *XhoI* site close to the right-border repeat of the T-DNA for identification. One single-copy line containing the full T-DNA insertion with the *XhoI* site was chosen for further study. Thermal asymmetric interlaced (TAIL)-PCR was performed and sequence analysis showed that the T-DNA was inserted in the twelfth intron of the annotated gene At5g49570.1. The left-border repeat had remained intact, except for the outermost two G-residues. Of the right-border repeat, only two G-residues remained. Based on the sequence data, we designed primer r4, which is complementary to a short stretch of plant DNA sequence, the T-DNA junction, and the *XhoI* site of the T-DNA for later analysis of RMCE events (Fig. 1, A and D).

Exchange T-DNA as a Circular Substrate for Cre

Single-stranded T-DNA can become double stranded extrachromosomally to undergo RMCE with the target locus directly. However, it is also possible that T-DNA integrates randomly prior to RMCE with the target locus. In both cases, simultaneous *loxP/loxP* and *lox1571/lox1571* recombination events are required. We were interested in whether circularization could increase RMCE efficiency. To test whether circularization of exchange T-DNA pSDM3732 occurred in planta, the following experiment was performed. Arabidopsis C24 roots were transformed with *A. tumefaciens* carrying exchange T-DNA pSDM3732 in the presence of a *cre*-delivering strain. After 1 d of cocultivation, roots were harvested and chromosomal DNA was isolated. Cre-mediated recombination at the *loxP* sites in pSDM3732 will result in a circular molecule that can be detected using primers n1 and t3 (Fig. 1C). A 0.93-kb fragment was detected and directly sequenced. The

Figure 1. (Continued.)

signals in the different reading frames are included to prevent *nptII* expression from neighboring sequences upon random integration. The conditional negative selectable marker *codA* with the 34S promoter is placed outside the cassette. Arrow 1 indicates direct cassette exchange via a double crossover reaction *loxP* \times *loxP* and *lox5171* \times *lox5171*, mediated by Cre that is provided via a cotransforming T-DNA. C, Exchange T-DNA pSDM3732 contains an additional *loxP* site near the right-border repeat. Arrows marked with a 2 indicate cassette exchange via a Cre-mediated circularization step of the exchange T-DNA prior to insertion at the target site (A) and resolution of the inserted exchange cassette. Circle formation can occur prior to or after random integration of pSDM3732. One of the two hypothetical exchange reactions is drawn for circularized exchange T-DNA pSDM3732 via insertion at the *loxP* site, followed by resolution at the *lox5171* site. D, Result of precise cassette exchange (with either exchange construct pSDM3327 [B] or pSDM3732 [C]). The restored *nptII* selectable marker allows selection for correct cassette exchange using kanamycin. Large black arrows, *loxP* sites; large dashed arrows, mutant *lox* sites, *lox5171*; the direction of arrowheads in the boxes indicates promoter regions; small white triangles, *A. tumefaciens* left- and right-border repeats. For the Southern strategy, probes are drawn as gray boxes above the constructs; N, *NsiI*; P, *PstI*; S, *SalI*; X, *XhoI*; the expected fragment length (kb) is indicated above the lines. Beneath the constructs, white arrows indicate primer binding sites, the expected size of the PCR fragments is given, with the RMCE-PCR product from primers p1 and r4 indicated with a dashed line. E, Nucleotide sequence of the DE35S-*loxP*-*bar* fusion of the target cassette and the *loxP*-*nptII* fusion of the exchange cassette. Upon cassette exchange, *nptII* expression is initiated at the transcription and translation signals of the DE35S promoter, located outside the cassette. Restriction endonuclease recognition sites used for cloning are indicated. *loxP* sites are framed and their orientation is indicated with an arrow. The translational start codon ATG at the *NcoI* site for *bar* and *nptII* is framed.

fragment contained the expected sequences for precisely recombined *loxP* sites (and not border fusion or tandem T-DNA structures), showing that indeed Cre-mediated circle formation occurs.

Stability of the Exchange DNA in the Presence of Cre

The exchange cassette contains the *loxP* site and the heterospecific mutant *lox* site 5171 in inverted orientation. Initially, Lee and Saito (1998) reported that there was no detectable cross-recombination between these two sites. Later, a low level of recombination between these sites was observed using a sensitive assay in *Escherichia coli* (Siegel et al., 2001). We observed some instability of the exchange T-DNA when present in *A. tumefaciens* containing a *cre* expression plasmid (data not shown). To avoid unsolicited recombination of the exchange vectors in *Agrobacterium* prior to T-DNA transfer, we performed cocultivations with target plant material using two *Agrobacterium* strains: one providing the exchange cassette and one providing the *cre* expression unit.

RMCE Experiments

Roots from homozygous (BB) or hemizygous (B–) target plants were cocultivated with two *Agrobacterium* strains: the first carrying one of the two exchange T-DNAs and the second harboring binary vectors with a *cre* cassette driven either by the nopaline synthase (*nos*) promoter or the BigMac promoter as indicated in Table I. Control experiments were performed with the exchange T-DNA-providing strain only.

In six independent RMCE experiments (Table I), a total of 50 kanamycin-resistant calli were obtained (ranging from 1/132 to 1/755 calli/root explant). When Cre was not provided during cocultivation, similar numbers of kanamycin-resistant calli were, however, obtained (data not shown). This suggests that translational fusions of the *nptII* gene with endoge-

nous plant DNA sequences were formed, even though in the exchange T-DNA the *nptII* coding region is preceded by translational stop codons in all frames and the *nos* terminator signal to avoid such fusions. These fusions might have been the result of infrequent integration of exchange T-DNAs in which the left border had been truncated, resulting in loss of the stop codons. Plants that were regenerated from these control calli, however, had progeny that grew very poorly on kanamycin-containing medium, suggesting that the *nptII* expression level was low or cell type specific. In contrast, progeny of putative recombinant plants grew well on kanamycin-containing medium.

For comparison, random integration of a pDE35S-*loxP-nptII* control T-DNA after cocultivation with LBA1100 (pSDM3066) was obtained with an efficiency varying from 0.5 to 1 callus per root explant (data not shown).

PCR Analysis Indicates a High Percentage of RMCE

To identify *loxP* crossover events, chromosomal DNA of kanamycin-resistant calli was analyzed by PCR using primer set p1 (annealing to the DE35S promoter sequence of the target site) and n1 (annealing to the *nptII* sequence of the exchange cassette; Fig. 1D). Kanamycin-resistant calli derived from cocultivation experiments with the exchange vector in the absence of Cre did not contain the DE35S-*loxP-nptII* junction, but 37 (74%) of the kanamycin-resistant calli derived from cocultivations in the presence of Cre amplified a DE35S-*loxP-nptII*-specific junction indicative of site-specific recombination (data not shown). Subsequently, DNA samples in which a DE35S-*loxP-nptII* junction had been detected were screened for the presence of the *lox5171* junction using primer g1 (annealing to the *nptII-gus* exchange cassette) and primer r4 (the right-border junction of the target DNA; Fig. 1D). Thirty-three of 37 (89%) samples were positive for the *lox5171* junction (data not shown), suggesting correct

Table I. Efficiency of RMCE after transformation of *Arabidopsis* target plant 3164 with Cre and exchange T-DNA constructs

Experiment	Target Plant <i>bar</i> Locus ^a	Cre Construct ^b	Exchange T-DNA Construct	No. of Root Explants ^c	No. of Kanamycin-Resistant Calli	RMCE Events ^d	RMCE per No. of Kanamycin-Resistant Calli ^e
1	BB	nos-cre	3327	1,557	6	3	0.50
2	BB	nos-cre	3327	1,363	4	1	0.25
3	BB	nos-cre	3327	847	5	3	0.60
		bm-cre	3327	860	3	2	0.67
4	B–	nos-cre	3327	664	5	2	0.40
		bm-cre	3327	755	1	1	1.00
5	B–	nos-cre	3327	1,725	8	4	0.50
		nos-cre	3732	1,705	8	3	0.38
6	B–	nos-cre	3327	1,080	6	2	0.33
		bm-cre	3327	977	2	0	0
		nos-cre	3732	1,030	2	1	0.50
		bm-cre	3732	1,021	0	0	0

^aHemizygous (B–) or homozygous (BB) plant material. ^bbm, BigMac promoter. ^cRoots were cut into explants of 2 to 3 mm in size; up to 500 explants were plated per petri dish. ^dRMCE events determined by PCR using primers p1 and r4. ^eRMCE events per no. of kanamycin-resistant calli.

RMCE had occurred in these calli. To confirm that both recombinant *lox* junctions were physically linked, a PCR reaction with primer set p1/r4 was conducted. PCR conditions were optimized with respect to primers, polymerase, Mg²⁺ concentration, and amount of template to detect the 6.2-kb fragment indicative of RMCE. The 6.2-kb RMCE-PCR product was detected in 22 (67%) of 33 calli for which both *loxP* and *lox5171* crossover sites were detected. Difficulties with RMCE-PCR may have resulted in underestimation of the actual number of positive reactions. Figure 2 shows a gel of a typical PCR experiment in which several samples scored negative for RMCE-PCR (experiment 3, lane 5; experiment 6, lanes 1 and 4), but were confirmed positive using different DNA concentrations in later PCR reactions. In experiments that used homozygous target plants, a 1.2-kb PCR fragment is also expected if only one of the two alleles has undergone RMCE (Fig. 2, experiment 3). For most of the calli derived from hemizygous starting material, both a 1.2- and a 6.2-kb fragment were, however, also observed. This indicated that the DNA material used for PCR was likely obtained from chimeric callus that was derived from both target and recombinant cells. Summarizing, our PCR data suggest that in 44% (22 of 50) of the kanamycin-resistant calli identified, an RMCE event at the target locus had occurred.

RMCE Events Detected by Southern-Blot Analysis

The majority of kanamycin-resistant calli regenerated plants (R1). Offspring of six R1 plants obtained from independent calli were further subjected to extensive Southern-blot analysis. These R1 plants include the numbers 51N2, 51N3, 51N5, 52N1, 52N3, and 41B, in which the first identifier refers to the experiment, the second identifier indicates the used

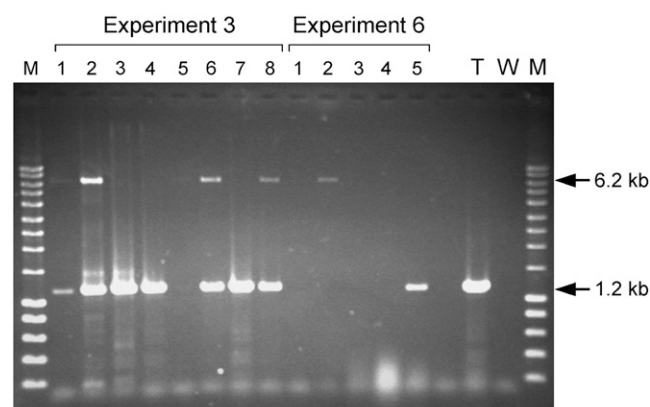


Figure 2. PCR analysis to detect correct RMCE. Shown is the ethidium bromide-stained gel from a typical RMCE-PCR analysis with primers p1 and r4, detecting the *bar*-target cassette (1.2 kb) and the exchanged cassette (6.2 kb) in chromosomal DNA of kanamycin-resistant calli of experiments 3 and 6. M, Molecular weight marker (1 kb; Smartladder, Eurogentec); T, callus of target plant; W, water control. Sample 7 is derived from cocultivation in the absence of Cre.

exchange cassette (1 = pSDM3327; 2 = pSDM3732), N indicates the use of *nos-cre*, B indicates cotransformation with *BigMac-cre*, and, when more than one callus was identified, the last identifier indicates the callus number from which the plants originated. All six R1 plants originated from transformation experiments using hemizygous target plants so as not to further complicate Southern analysis. PCR had indicated that an RMCE event had occurred as demonstrated by the 6.2-kb fragment amplified with primers p1 and r4, except for 52N1, which showed the presence of both *loxP* and *lox5171* junctions, but failed to show the correct RMCE fragment. We included this line to be able to confirm our PCR data. Seeds from the R1 plants were germinated on medium containing kanamycin. Their progeny segregated in a ratio of 3:1 (resistant:sensitive) as expected. Progeny plants were diploid, as determined by flow cytometry. Kanamycin-resistant R1 progeny were grown further and analyzed independently by Southern-blot analysis to be able to detect segregation of possibly integrated random exchange cassette or *cre* DNA sequences.

Correct RMCE is detected by a 7.0-kb *Sall*-*Xho*I fragment (Fig. 1D) hybridizing with the 35S probe. This product was found for all analyzed offspring of five lines (Fig. 3A), but not for 52N1 as expected from the PCR results. For the target cassette, this *Sall*-*Xho*I digest yielded the expected 1.3-kb *Sall* fragment (Figs. 1A and 3A, lane T). RMCE was further confirmed with an *Nsi*I digest hybridized with the 35S probe (Fig. 3B). Digestion of target plant DNA with *Nsi*I yields a 5.7-kb fragment (Figs. 1 and 3B, lane T). Accurate RMCE will thus yield a 10.8-kb *Nsi*I fragment (Fig. 1D), which was indeed detected for all lines except 52N1. Hybridization of the *Nsi*I blot with the *gus* probe (Fig. 3D) cemented our conclusion that correct RMCE had occurred in the five lines that were preselected as RMCE events based on PCR; similar banding patterns were observed as in the *Nsi*I/35S blot (Fig. 3B), except for the target line (lane T) in which the 5.7-kb 35S fragment did not hybridize with the *gus* probe as expected, and in lane 17 (41B) in which the largest fragment detected in the *Nsi*I/35S blot did not hybridize with the *gus* probe (Fig. 3D). This larger fragment originates from a random insertion of the pBigMac-*cre* construct pSDM3088 in 41B, which is detected using a 35S probe due to cross-hybridization with the BigMac promoter. In all other lines, the *nos-cre* construct was used during transformation and not detected using a 35S probe.

A *Pst*I digest was hybridized with the 35S probe that allows the simultaneous detection of both the *loxP* (1.2 kb) and *lox5171* (8.0 kb) recombinant junctions, although the presence of both fragments does not confirm they are physically linked, as already shown in previous blots (Figs. 1D and 3E). In the target line, a 5.7-kb fragment was found as expected. Indeed, all lines showed evidence of correct recombination of both junctions. Interestingly, line 52N1, in which we were unable to detect a correct RMCE fragment, did contain both recombinant junctions, confirming earlier

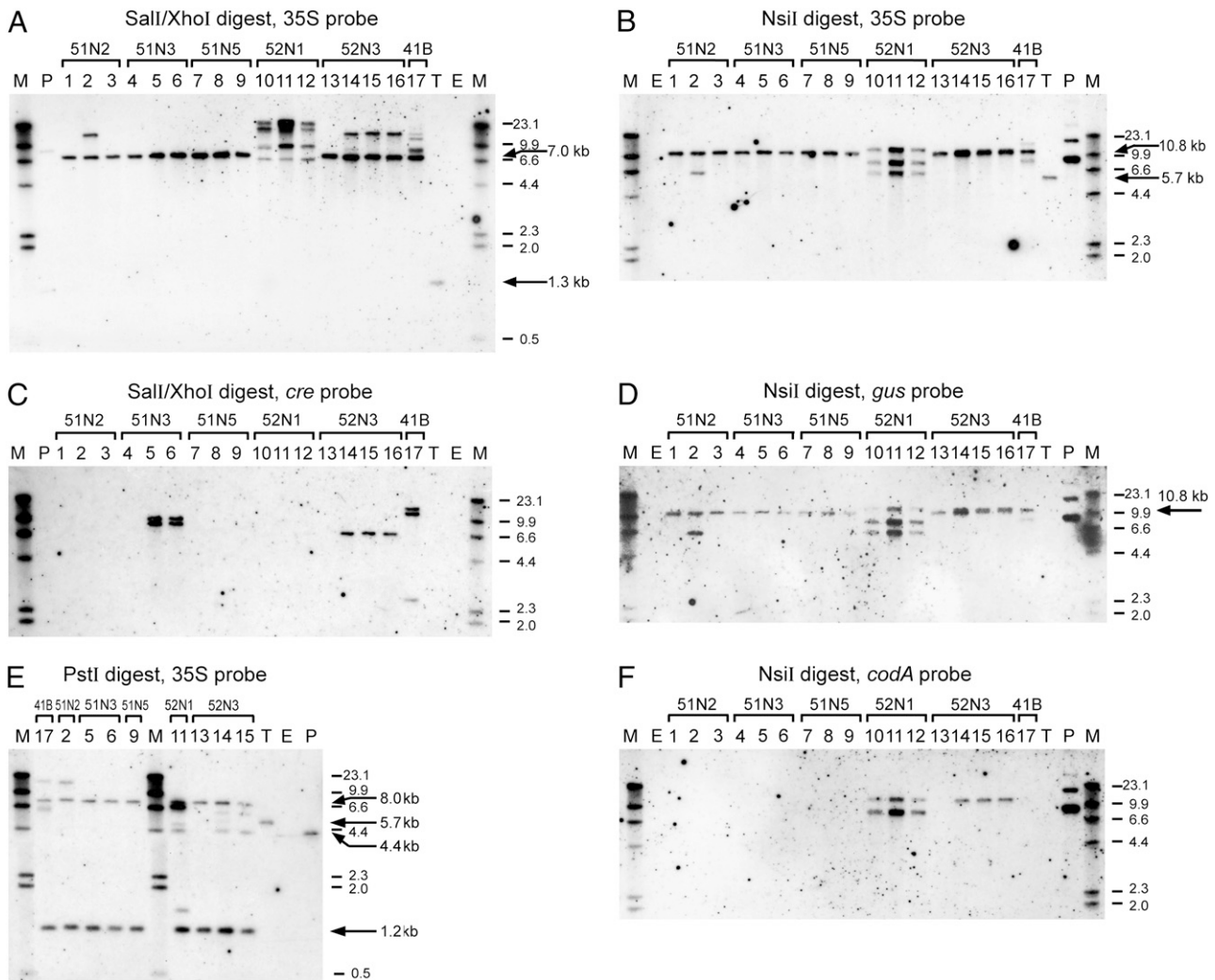


Figure 3. Southern-blot analyses of putative RMCE lines. Chromosomal DNA of target plant (T), untransformed control ecotype C24 (E), and plants of putative RMCE lines derived from experiments 4 and 5 was either digested with *NsiI* (B, D, and F) or *PstI* (E) or double digested with *Sall* and *XhoI* (A and C), and hybridized with 35S (A, B, and E), *cre* (C), *gus* (D), or *codA* (F) probes, respectively. Codes above the horizontal brackets indicate the name of the parent plant line of which the individuals in the lanes are derived. Numbering of the individuals is consistently used for all blots. The name of the line indicates the following. First identifier, Experiment number; second identifier, 1 for exchange T-DNA 3327 or 2 for T-DNA 3732; third identifier, N for T-DNA containing *pnos-cre* or B for *pBigmac-cre*. When more than one callus was selected, the last identifier indicates the callus number of which the plants originated. Individual lanes underneath a bracket show different kanamycin-resistant progeny plants from a single regenerated R1 shoot. Lane P contains digested plasmid DNA of exchange construct pSDM3327 mixed with digested C24 chromosomal DNA; M, DIG-labeled molecular weight marker. Arrows indicate the expected RMCE fragment 7.0 kb and the target fragment 1.3 kb (A); the expected RMCE fragment 10.8 kb and the target fragment 5.7 kb (B); the expected 10.8-kb RMCE fragment (D); and CE fragments of 1.2 kb and 8.0 kb, the target fragment 5.7 kb, and the 4.4-kb fragment specific for exchange T-DNA, not recombined at the *lox5171* site (E).

PCR analysis. Summarizing, these data clearly show that the five R1 plants identified on the basis of RMCE-PCR are indeed the result of correct RMCE events.

Analysis of Additional T-DNA Integration Events

We analyzed the presence of additional random insertions for the five lines that resulted from a correct RMCE event. Southern-blot analysis revealed that in three of five analyzed recombination events (51N3,

52N3, and 41B), the *cre* gene, which was cotransformed with the exchange cassette, was also integrated in the plant genome (Fig. 3C). The *cre* DNA sequence does not contain *Sall* or *XhoI* sites, allowing us to estimate the number of T-DNA insertions. For lines 51N3 (two insertions) and 52N3 (one insertion), the *cre* insertions were able to segregate away from the RMCE locus (Fig. 3C, lane 4 of line 51N3 and lane 13 of line 52N3). We cannot comment at this stage on segregation of the *cre* gene for line 41B because we only analyzed one

offspring plant for this recombinant. Our data clearly show that a strategy with transiently provided Cre on a cotransforming T-DNA is effective and can result in easy identification of stable RMCE plants.

Random integration of the exchange T-DNA was examined by hybridization of the *NsiI* blot with a *codA* probe (Fig. 3F). Random integration of complete exchange T-DNAs of pSDM3327 or pSDM3732 will result in detection of an 8.2-kb fragment (Fig. 1B). In these experiments, we did not apply selection against expression of *codA*. Surprisingly, only one of the five lines showed a signal when hybridized with the *codA* probe. For line 52N3 in three of the four analyzed R2 plants, a *codA* hybridizing fragment coincidentally of similar size as the 10.8-kb RMCE fragment (Fig. 3, B and D) was detected, indicating that exchange T-DNA had inserted also randomly. The fact that the fragment is not of the expected size can easily be explained by truncation of the T-DNA, resulting in the loss of an *NsiI* site at one of the distal ends of the T-DNA. *Sall/XhoI* and *PstI* digests allowed simultaneous visualization of RMCE and random inserts in this line 52N3 using a *35S* probe. In agreement with the *codA* blot, in the same offspring plant of 52N3 (lane 13 in all blots), no random fragment is detected (Fig. 3, A and E). Figure 3C shows that the *cre* gene is also absent in this offspring plant, resulting in a clean single-copy RMCE event. For lines 51N2 and 41B, however, additional hybridizing fragments were detected using *35S* and *gus* probes. 51N2 contains one additional insert that hybridizes with *gus* and *35S* probes (*Sall/XhoI*, *NsiI*, and *PstI* digest). This T-DNA could clearly segregate from the RMCE locus because it was only detected in one of the three analyzed plants, again resulting in a clean single-copy RMCE recombinant. 41B is more difficult to interpret because the *35S* probe hybridizes with the RMCE fragment, with two copies of the randomly integrated *cre* gene (Fig. 3C) as well as the randomly integrated exchange cassette. However, the data also show that one additional copy of the exchange cassette had integrated as deduced from the blot hybridized with a *gus* probe. The absence of *codA* and fragment sizes that differ from the expected sizes for random integration is consistent with integration of truncated T-DNAs of the exchange vector in 51N2, 52N3, and 41B.

Summarizing, line 51N5 was the result of a clean RMCE event, with no additional insertions of *cre* or exchange vector DNA. Line 51N3 contained additional *cre* insertions that could be segregated away easily. Similarly, recombinant 51N2 contained an additional random T-DNA insert that was lost in part of the offspring. Although we detected random insertions of *cre* and exchange vector in plants of lines 41B and 52N3, clean RMCE offspring could easily be obtained for such lines as shown for 52N3.

DISCUSSION

Transgene integration in plants, independent of the transformation method used, is beyond our control

and takes place at random, unknown positions in the genome. Because directed integration by homologous recombination is extremely inefficient, site-specific recombination strategies have been developed to target transgenes to predetermined genomic locations. Targeted integration is a useful tool to eliminate transgene instability and variation in gene expression caused by transgene integration position and integration patterns and provides a means to obtain stable transgenic plants. The development of efficient methods for site-specific integration, using different protocols for transformation, is important for both fundamental research and crop improvement. Several groups have shown that, indeed, targeting to specific predetermined genomic locations, both in mammalian cells as well as in plants, resulted in reproducible gene expression levels (Fukushige and Sauer, 1992; Feng et al., 1999; Srivastava et al., 2004; Chawla et al., 2006). Chawla et al. (2006) showed that consistent gene expression levels were obtained in single-copy rice lines during several generations and that an allelic gene dosage effect nearly doubled gene expression levels. An additional advantage of directed integration is the elimination of unwanted mutations due to random insertion of transgenes in essential genes or essential genomic regions. Here, we provide evidence for successful targeting using a Cre-based RMCE approach. This strategy uses *Agrobacterium*-mediated transformation and is based on selection for kanamycin resistance as a result of precise exchange of a T-DNA cassette with a genomic target cassette that was placed in the genome via a first round of transformation. Introduction of target sites at particular genomic locations, for instance, via homologous recombination, is difficult. However, randomly introduced good target sites can be preselected based on expression levels and analysis of the integration site, allowing efficient future targeting to such a site.

The natural gene delivery system of *Agrobacterium* is a preferred method for the transformation of a wide range of plants. As a consequence of the highly efficient random T-DNA integration process, a tight selection system is required for identification of site-specific, single-copy integration events. Here, we used a promoter trap strategy that targets a promoterless *nptII* marker that will become expressed after specific integration downstream of a promoter sequence present at the target site. Stop codons in three reading frames were introduced upstream of the *lox-nptII* coding region to further diminish selection of unwanted random integration events as a result of fusion to endogenous DNA promoter sequences. In several small-scale RMCE experiments (each up to four plates with small, excised root explants) in which *cre* T-DNA was provided by a second *Agrobacterium* strain, we identified a total of 50 kanamycin-resistant calli. Using PCR analysis, which detected the fully exchanged cassette, we showed that 44% of these kanamycin-resistant calli carried putative RMCE events, indicating the stringency of the selection procedure. The

recombinant nature of offspring obtained from five of these events was confirmed by Southern analysis.

Different site-specific recombination systems, namely, *R/RS* and *Cre/lox*, have been used in plants to obtain site-directed integration. Integration at a single genomic recombination site in the genome requires circular double-stranded DNA also harboring a single compatible recombination site. Plasmid DNA, or any double-stranded DNA that can be circularized upon entry in the host cell by a recombinase-mediated reaction to prevent integration of vector sequences (Srivastava and Ow, 2002), is a good substrate to be delivered via direct DNA transfer methods. A protoplast-based transformation protocol for tobacco has been used successfully for site-specific integration (Albert et al., 1995); however, protoplast-dependent transformation protocols are not widely applicable. Particle bombardment is a preferred method for transformation of cereals (Christou, 1995) and site-specific integration in rice using biolistics has been successful using a combination of mutant *lox* sites and disruption of *cre* expression (Srivastava and Ow, 2002; Srivastava et al., 2004). The single-stranded linear T-DNA transferred by *Agrobacterium*, however, is in principle a poor substrate for a site-specific integration reaction. In addition, *Agrobacterium* T-DNA has evolved to integrate highly efficiently into the genome at random positions. It is therefore not surprising that direct DNA transfer methods result in much higher ratios of site-specific to random integration (Albert et al., 1995) than *A. tumefaciens*-based methods. The efficiency of RMCE in this study, compared to random integration of control T-DNA, was on the order of 0.3%. However, we detected total numbers of integrants at the same order of magnitude as that described after bombardment of rice (Srivastava and Ow, 2002; Srivastava et al., 2004). Nanto et al. (2005) and Nanto and Ebinuma (2007) applied an RMCE strategy in plants and introduced an exchange T-DNA cassette using the *R/RS* recombinase system in tobacco. These authors used a completely different strategy than reported here, which was based rather on the accumulation of exchange events after a prolonged time of selection for rooted shoots (up to 6 months). Whereas here we used a promoter trap strategy to preferentially select kanamycin-resistant calli containing recombination events that occurred at the target site only, Nanto and colleagues used an *hpt* expression cassette on the exchange T-DNA, also allowing the selection of hygromycin-resistant calli due to random integration events. Due to the high efficiency of *Agrobacterium*-mediated random T-DNA integration, RMCE in this case is therefore most likely due to exchange with prior randomly integrated copies. The rationale for identification of single-copy RMCE events was the specific placement of three identical *RS* sites on the exchange T-DNA, containing the *RS*-flanked *hpt* exchange cassette and the *RS*-flanked *R* and *ipt* genes, combined with the use of the *ipt* gene as a negative selectable marker. RMCE events were detected among rooting shoots with a normal phenotype, which indi-

cated the *R*-mediated deletion of the *ipt* and linked *R* gene, as well as nontruncated random events. Comparison of the efficiency of different existing protocols for site-specific integration is difficult due to the use of different strategies, DNA transfer methods, and selection procedures. The high efficiency of the *Agrobacterium* transfer method and the ease of the procedure, combined with a stringent selection system, make our *Agrobacterium*-RMCE-based site-specific integration method a very simple and practical procedure.

Despite the fact that T-DNA may not be a perfect substrate for site-specific integration, it was shown that *A. tumefaciens* T-DNA could be successfully targeted to a single genomic *lox* site (Vergunst and Hooykaas, 1998; Vergunst et al., 1998b) or via RMCE (Nanto et al., 2005; Nanto and Ebinuma, 2007; this article). The single-stranded nature of T-DNA may not be a problem because it was demonstrated that single-stranded T-DNA can become double stranded extrachromosomally and genes located on T-DNA become expressed and recombine efficiently among each other (Ofringa et al., 1990; Narasimhulu et al., 1996; Tzvira et al., 2003; Marillonnet et al., 2004). In addition, efficient selection of T-DNA targeted to a single genomic *lox* site was obtained by placing an additional *lox* site on the T-DNA integration vector to allow Cre-mediated circularization of the T-DNA prior to site-specific integration (Vergunst et al., 1998b). This circularization could have occurred prior to or after random integration. RMCE, in principle, does not require circularization of the exchange cassette. We anticipated that in an RMCE strategy circle formation prior to exchange might also be advantageous. Therefore, we tested whether placing an additional *loxP* site adjacent to the opposite T-DNA border sequence to allow circularization prior to or after random integration of the exchange T-DNA would improve RMCE frequency. Sequence analysis showed that correct circularization at the *loxP* sites in the exchange DNA after cotransformation with *cre* T-DNA to wild-type root explants had occurred. However, comparison of the efficiencies of RMCE after transformation with an exchange T-DNA with or without an additional *loxP* site for circularization did not show any difference (Table I, experiments 5 and 6). Circle formation in this strategy, therefore, did not improve RMCE frequency.

Site-specific integration strategies, using any transformation method, require that recombinase activity is controlled in some way, which can be combined with the use of specific mutant *lox* sites to prevent reversible recombination events. A single *lox*-targeting strategy involving displacement of *cre* from its promoter turned out to be insufficient to completely stabilize T-DNA targeting events (Vergunst et al., 1998b). To increase stability of integrants, here, Cre was provided from cotransferred T-DNA, combined with an RMCE strategy using incompatible *loxP* and *lox5171* sites (Lee and Saito, 1998) that flanked both the target cassette and the exchange cassette in inverted orientation. Siegel et al. (2001) showed, however, that

incompatibility between *loxP* and *lox5171* sites was not complete and we indeed observed some instability between the *loxP* and *lox5171* sites of the exchange cassette in the presence of Cre in *A. tumefaciens* and *E. coli*. Therefore, we physically separated the targeting construct and the *cre* expression construct by using two *Agrobacterium* strains to deliver T-DNAs. Highly efficient delivery of two different T-DNAs into the same cell has been described. In *Nicotiana benthamiana*, cotransfer efficiencies of up to 90% were detected in infiltrated leaves using nonselectable markers (Marillonnet et al., 2004). De Buck and colleagues showed, using a different approach in Arabidopsis, that in 50% of selected transformants a second T-DNA was transiently expressed (De Buck et al., 2000). Here, genuine cassette exchange was mediated by transiently expressed *cre* T-DNA. From detailed Southern-blot analysis of five RMCE events, we concluded that two of these lines indeed contained the correctly recombined exchange cassette without additional *cre* sequences inserted in the genome. For three other RMCE events, the *cre* gene was detected in the genome, but in at least two of these lines, *cre* segregated independently from the RMCE locus in the offspring. This allowed recovery of RMCE plants free of *cre* sequences.

In our strategy, we included the possibility of selecting against random integration of the exchange T-DNA by placing the *codA* gene adjacent to the exchange cassette. Selection for absence of *codA* was, however, not applied because limited numbers of kanamycin-resistant calli were already obtained. An advantage of omitting selection for loss of *codA* was that an offspring plant with a clean single-copy RMCE event could now be selected after segregation of *codA* sequences in the offspring, which otherwise would have been lost. Unexpectedly, two lines (51N2 and 41B) that did not contain *codA* sequences contained randomly integrated DNA shown by detection with *gus* and 35S probes. It might be that two copies of the exchange T-DNA integrated as an inverted repeat linked at their right borders. Excisional recombination by Cre would then lead to loss of both *codA* sequences. It is difficult to establish whether RMCE occurred directly with extrachromosomal T-DNA that had become double stranded or with randomly integrated copies of the exchange T-DNA. Any footprints, such as *codA* sequences, that may be left after prior random integration may equally have been the result of integration after extrachromosomal recombination of T-DNA.

In summary, among the five analyzed putative RMCE events, one clean RMCE event without additional *cre* or exchange T-DNA insertions (51N5) was obtained. In three other RMCE events, the randomly integrated T-DNA sequences easily segregated, resulting in clean RMCE events. The simplicity of our RMCE strategy, the stringency of selection, and the ease of detection of RMCE by PCR provide good potential to select target lines with high expression loci and to target transgenes to predetermined genomic positions

to improve the repeatability of transgene expression levels.

MATERIALS AND METHODS

Bacterial Strains

Agrobacterium tumefaciens strain LBA1100 (C58C1 with a disarmed octopine-type pTiB6 plasmid; Beijersbergen et al., 1992) was used for RMCE and plant transformation experiments. *Escherichia coli* DH5 α was used for cloning.

Recombinant DNA Techniques

Standard cloning techniques were carried out according to Sambrook et al. (1989). Restriction enzymes were purchased from New England Biolabs. Bacteria were grown in Luria culture medium (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ yeast extract, 8 g L⁻¹ NaCl), with appropriate antibiotics. Antibiotics (Duchefa Biochemie BV) were used at concentrations of 100 mg L⁻¹ kanamycin, 20 mg L⁻¹ rifampicin, 250 mg L⁻¹ spectinomycin, and 40 mg L⁻¹ gentamycin for *A. tumefaciens*. For selection in *E. coli*, 100 mg L⁻¹ carbenicillin, 10 mg L⁻¹ gentamycin, or 25 mg L⁻¹ kanamycin were used.

Plasmid Constructs

Cre expression vector pSDM3088 (*pBigMac-cre*) was described previously (Vergunst and Hooykaas, 1998) and contains the *cre* gene driven by the strong *BigMac* promoter placed between the T-DNA border repeats. A second *cre* expression vector, pSDM3021 (*pnos-cre*), was made as follows. An *XhoI-EcoRI* fragment containing a mannopine synthetase (*mas*) termination signal and a polylinker containing a multiple cloning site (MCS; 5'-GTCGACAAGCTTGGGAAGATCTAGTACTTTGGGGTACCCCGCTCTAGAGCGAATTC [SalI and EcoRI sites underlined]) were inserted in the *XhoI/SalI*-digested pUC21. The *nos* promoter region of the 35S-*lox-cre* target plasmid (pSDM3110) described earlier (Vergunst et al., 1998b) was then inserted in the *BglIII/HindIII* sites of the MCS, resulting in pSDM3018. The *nos*-MCS-*mas* cassette was cloned between the T-DNA borders of binary vector pSDM14 (Offringa, 1992) as a *SalI-XhoI* fragment, resulting in pSDM3019. Finally, the *cre* coding region of pUC19CRE (Mozo and Hooykaas, 1992) was cloned in the *EcoRI/XbaI* sites of the MCS of pSDM3019 after the *SphI* site that created an out-of-frame ATG just upstream of the ATG start codon in pUC19CRE had been removed (Klenow fragment of T4 DNA polymerase) via a subcloning step in pC19H. This resulted in pSDM3021, harboring a *pnos-cre-tmas* cassette.

The structure of the T-DNA of vector pSDM3164 that was used to produce the target line is drawn in Figure 1A. The sequence of the DE35S-*loxP-bar* translational fusion is depicted in Figure 1E (top) and was constructed from the following sequences: As a source for the promoter-ATG-*loxP* fusion, we used a fragment of the *p35S-ATG-lox-npt* control vector (Vergunst and Hooykaas, 1998), which contains the CaMV 35S promoter sequence with a double enhancer and alfalfa mosaic virus (AMV) 5'-leader sequence (DE35S) transcriptionally fused to *loxP*. To be able to create an in-frame translational fusion with a *bar-tnl* sequence present on a (partial) *BglIII* fragment of excision vector pSDM3043 (Vergunst and Hooykaas, 1998), a *BglIII* linker was added first that resulted in a frame shift and an additional *Clal* site. This yielded the DE35S-*loxP-bar-tnl* construct pSDM3145. Two oligos representing the *lox5171* site (Lee and Saito, 1998) were annealed and subcloned as an *XbaI-StuI* fragment into pUC28 (pSDM3150), followed by ligation into vector pSDM3145, resulting in pSDM3162. Finally, the DE35S-*loxP-bar-tnl-lox5171* construct was cloned between the right- and left-border repeats of binary vector pSDM14 (Offringa, 1992), yielding pSDM3164.

The structure of the exchange T-DNA of vector pSDM3327 is depicted in Figure 1B, which was constructed from the following DNA fragments: A *loxP* site was cloned via multiple cloning steps to the *nptII* open reading frame devoid of the start codon (Fig. 1E, bottom). *nptII* with the octopine synthase transcription termination sequence originated from pSDM56 (De Groot, 1992). The *nos* transcription terminator region of pSDM3322 was introduced as an *EcoRI-SacI* fragment and further cloned to binary vector pSDM3014, which is a derivative of pSDM14 in which the *NotI* site was deleted by filling in the site using T4-DNA polymerase. To introduce three stop codons, a *BamHI-KpnI* linker was placed in the MCS preceding the *nos* terminator region yielding sequence 5'-GTCGACGGATCCTAGTCTAGACTAGGTACC in construct

pSDM3325. The *lox5171* site of pSDM3150 was cloned to the *codA* gene under control of the figwort mosaic virus 34S promoter with the CaMV 35S 3'-region, a derivative of pSDM5042 (Schlaman and Hooykaas, 1997). The *codA* expression cassette-*lox5171* was cloned as an *XhoI*-*NotI* fragment into pSDM3325, yielding pSDM3159. pSDM3330 contains the *gus* gene under the control of the *BigMac* promoter and mannopine synthetase (*mas*) transcription terminator region of pGN7344 (Comai et al., 1990) and was cloned as a *NotI* fragment into the *NotI* site of pSDM3159, yielding pSDM3327.

The structure of the exchange T-DNA of vector pSDM3732 is depicted in Figure 1C. An additional *loxP* site was cloned as a *SalI* fragment of pMS103 (Snaith et al., 1995) into the *XhoI* site near the right border of pSDM3327 in direct orientation to the *loxP* site to allow Cre-mediated circularization of the exchange T-DNA prior to RMCE.

The control T-DNA vector pSDM3066, containing pDE35S-*ATG-loxP-nptII*, was described previously (Vergunst and Hooykaas, 1998). Target and exchange T-DNA constructs were verified by restriction enzyme analyses and sequencing.

Plant Material and Transformation

Arabidopsis (*Arabidopsis thaliana*) ecotype C24 and transgenic lines thereof were used. Greenhouse and tissue culture conditions and the *Agrobacterium*-mediated transformation protocol of *Arabidopsis* root explants were described by Vergunst et al. (1998a). Selection of target plant lines based on resistance for PPT (Duchefa Biochemie BV) followed the description of Vergunst et al. (1998a). Single-copy transformants were identified on the basis of genetic and Southern-blot analyses and further analyzed for the presence of the complete *bar* cassette by Southern analysis using a *bar* probe. One transgenic line was selected and the DNA sequence of the T-DNA insert junction was determined using TAIL-PCR (Liu et al., 1995). The ploidy level of the progeny plants was determined by flow cytometry (Plant Cytometry Services). Homozygous T3 seeds were germinated in liquid medium containing 5 mg/L PPT and roots were excised for use in cassette-exchange root-transformation experiments. To generate hemizygous roots, the homozygous line was back-crossed, using the transgenic line as pollen donor, to the wild type. The resulting seeds were germinated in liquid medium, supplemented with 5 mg/L PPT.

Homozygous or hemizygous root explants were incubated with a mixture of two *Agrobacterium* strains, LBA1100 (exchange T-DNA) and LBA1100 (Cre-T-DNA) in a 1:1 ratio at a final concentration of $OD_{600} = 0.2$, prior to cocultivation for 3 d on callus-inducing medium agar plates (Vergunst et al., 1998a). In control cocultivations in the absence of LBA1100 (Cre-T-DNA), a final concentration of $OD_{600} = 0.1$ of LBA1100 (exchange T-DNA) was used.

Root explants, with a length of about 2 to 3 mm, were cultured on shoot-inducing medium with 50 mg/L kanamycin and 100 mg/L timentin (Vergunst et al., 1998a). Parts of the kanamycin-resistant calli were multiplied on callus-inducing medium and used for PCR analysis or for plant regeneration. The ploidy level of regenerated plants was determined using flow cytometry.

The random T-DNA integration frequency was estimated by cocultivation of target roots with LBA1100 (pSDM3066) constitutively expressing *nptII* in plant cells. The frequency of kanamycin-resistant green calli per root explant was determined 3 weeks after cocultivation. Kanamycin-resistant calli resulting from RMCE experiments were counted after 3 to 5 weeks.

DNA Isolation and Southern Analysis

Chromosomal DNA was isolated from 0.1 to 0.5 g of flower buds or leaves, using a Nucleon PhytoPure plant DNA extraction kit (GE) according to the manufacturer's protocol. Three micrograms of chromosomal DNA were digested with the appropriate restriction enzymes and separated on a 0.6% Tris borate/EDTA agarose gel. Twenty nanograms of digoxigenin (DIG)-labeled DNA molecular weight marker II, λ -DNA digested with *HindIII* (Roche Diagnostics), was used as a size marker. DNA blotting was performed on positively charged nylon membranes (Roche) and DIG dUTP was incorporated in the probes with the PCR DIG-labeling mix (Roche), according to the manufacturer's recommendations. Hybridization and immunological detection of the DIG signal (Neuhaus-Url and Neuhaus, 1993) was performed using the substrate CDP star (Roche).

PCR Analysis

Calli were cultured for at least 8 weeks on kanamycin-containing medium. Chromosomal DNA was isolated from callus or young leaves using a cetyl-

trimethyl-ammonium bromide method according to Lassner et al. (1989). PCR reactions were performed in a T1 thermocycler (Whatman/Biometra) with primers as indicated (Fig. 1). A standard PCR protocol of 30 cycles was used: 1 min at 95°C, 1 min with annealing temperature (T_A) of 55°C (or as indicated for specific reactions), and 1 min at 72°C. The reaction mixture (25 mL) contained 25 to 50 ng of template DNA, 0.4 μ M of each primer, 200 μ M dNTPs, and 0.5 units of RedTaq polymerase (Sigma) with the provided buffer.

PCR analysis to detect recombination site junctions used the following primers (5'-3'): *LoxP*-PCR, to detect the pDE35S-*loxP-nptII* junction site in the target locus, used primers p1 (GACGCACAATCCCCTATCCTCGCAA) and n1 (TGATATTCGGCAAGCAGGCATC) at a T_A of 64°C; and *Lox5171*-PCR, to amplify the *gus-lox5171*-right-border target plant junction, used primers g1 (CGCTGGACTGGCATGAAGTTC) and r4 (CCATGAGTGATTAATAGAAGT-CACACCTCGA) preceded by 10 touch-down cycles with a T_A 60°C to 55°C, changing 0.5°C per cycle.

Primer t3 (GGTTCTTATAGGGTTTCGCTCATGTGT) was used in combination with n1 at a T_A of 64°C to detect circle formation of T-DNA in plant cells.

To detect a 6.2-kb RMCE-specific fragment, the following protocol was developed: The PCR block was preheated to 98°C. A program of 35 cycles was preceded by a period of 4 min at 98°C. Cycles were composed of 45 s at 98°C, 30 s at 63°C, and 4 min at 72°C. The reaction mixture (50 mL) contained 25 to 100 ng of template DNA, 200 μ M dNTPs, 7.9 mM $MgCl_2$, 0.5 μ M of primers p1 and r4, and 0.6 units Phusion DNA polymerase (Finnzymes) in the provided GC buffer.

To amplify the probes for Southern-blot analysis, several primer sets were used: primers p3 (GTGGGATTGTGCGTCATCCC) and p5 (GGATTGATGT-GATAATTCGGATGGAGTC) were used to amplify the 35S probe; primers c3 (ACGCTGGTTAGCACCGCAGG) and c4 (CAGGCGCACCATGGCCCTG) to amplify the *cre* probe; and primers CD3 (CGCTCACCGTTGGGATACG) and p34S1 (CCGGTACAATAATGGGGAGG) for the *codA* probe; primers *gusU* (CAGCGAAGAGGCAGTCAACGGGGAA) and *gusL* (CATTGTTG-CCTCCCTGCTCGGTT) for the *gus* probe.

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LITERATURE CITED

- Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. *Plant J* 7: 649–659
- Baer A, Bode J (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr Opin Biotechnol* 12: 473–480
- Bakkeren G, Koukolikoff Z, Grimsley N, Hohn B (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules from whole plants early after transfer. *Cell* 57: 847–857
- Beijersbergen A, Den Dulk-Ras A, Schilperoort RA, Hooykaas PJJ (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. *Science* 256: 1324–1327
- Bouhassira EE, Westerman K, Leboulch P (1997) Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood* 90: 3332–3344
- Bundock P, Den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ (1995) Transkingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J* 14: 3206–3214
- Chawla C, Ariza-Nieto M, Wilson AJ, Moore SK, Srivastava V (2006) Transgene expression produced by biolistic-mediated site-specific gene integration is consistently inherited by the subsequent generations. *Plant Biotechnol J* 4: 209–218
- Chilton MD, Que Q (2003) Targeted integration of *Agrobacterium* T-DNA into the tobacco genome at double-strand breaks: new insights in the mechanism of T-DNA integration. *Plant Physiol* 133: 956–965
- Christou P (1995) Particle bombardment. *Methods Cell Biol* 50: 375–382
- Citovsky V, Guralnick B, Simon MN, Wall JS (1997) The molecular structure of *Agrobacterium* VirE2-single-stranded DNA complexes involved in nuclear import. *J Mol Biol* 271: 718–727

- Comai L, Moran P, Maslyar D** (1990) Novel and useful properties of a chimeric plant promoter combining CAMV-35S and Mas elements. *Plant Mol Biol* **15**: 373–381
- Corneille S, Lutz K, Svab Z, Maliga P** (2001) Efficient elimination of selectable marker genes from the plastid genome by the Cre-lox site-specific recombination system. *Plant J* **27**: 171–178
- Day CD, Lee E, Kobayashi T, Holappa LD, Albert H, Ow DW** (2000) Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. *Genes Dev* **14**: 2869–2880
- De Buck S, De Wilde C, Van Montagy M, Depicker A** (2000) Determination of the T-DNA transfer and the T-DNA integration frequencies upon cocultivation of *Arabidopsis thaliana* root explants. *Mol Plant Microbe Interact* **13**: 658–665
- De Groot MJA** (1992) Studies on homologous recombination in *Nicotiana tabacum*. PhD thesis. Leiden University, Leiden, The Netherlands
- Feng YQ, Seibler J, Alami R, Eisen A, Westerman KA, Leboulch P, Fiering S, Bouhassira EE** (1999) Site-specific chromosomal integration in mammalian cells: highly efficient Cre recombinase-mediated cassette exchange. *J Mol Biol* **292**: 779–785
- Francis KE, Spiker S** (2005) Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integration events. *Plant J* **41**: 464–477
- Fukushige S, Sauer B** (1992) Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. *Proc Natl Acad Sci USA* **89**: 7905–7909
- Gilbertson L** (2003) Cre-lox recombination: Cre-active tools for plant biotechnology. *Trends Biotechnol* **21**: 550–555
- Gleave AP, Mitra DS, Mudge SR, Morris BAM** (1999) Selectable marker-free transgenic plants without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. *Plant Mol Biol* **40**: 223–235
- Kim SI, Veena, Gelvin SB** (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. *Plant J* **51**: 779–791
- Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P** (2003) Transgene integration, organization and interaction in plants. *Plant Mol Biol* **52**: 247–258
- Kopertekh L, Juttner G, Schiemann J** (2004) PVX-Cre-mediated marker gene elimination from transgenic plants. *Plant Mol Biol* **55**: 491–500
- Lassner MW, Peterson P, Yoder JI** (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Mol Biol Rep* **7**: 116–128
- Lauth M, Spreafico F, Dethleffsen K, Meyer M** (2002) Stable and efficient cassette exchange under non-selectable conditions by combined use of two site-specific recombinases. *Nucleic Acids Res* **30**: e115
- Lee G, Saito I** (1998) Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216**: 55–65
- Liu YG, Mitsukawa N, Oosumi T, Whittier RF** (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J* **8**: 457–463
- Marillonnet S, Giritich A, Gils M, Kandzia R, Klimyuk V, Gleba Y** (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc Natl Acad Sci USA* **101**: 6852–6857
- Mozo T, Hooykaas PJJ** (1992) Design of a novel system for the construction of vectors for *Agrobacterium*-mediated plant transformation. *Mol Gen Genet* **236**: 1–7
- Nanto K, Ebinuma H** (2007) Marker-free site-specific integration in plants. *Transgenic Res*
- Nanto K, Yamada-Watanabe K, Ebinuma H** (2005) *Agrobacterium*-mediated RMCE approach for gene replacement. *Plant Biotechnol J* **3**: 203–214
- Narasimhulu SB, Deng XB, Sarria R, Gelvin SB** (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell* **8**: 873–886
- Neuhaus-Url G, Neuhaus G** (1993) The use of the nonradioactive digoxigenin chemiluminescent technology for plant genomic southern blot hybridization: a comparison with radioactivity. *Transgenic Res* **2**: 115–120
- Offringa R** (1992) Gene targeting in plants using the *Agrobacterium* vector system. PhD thesis. Leiden University, Leiden, The Netherlands
- Offringa R, De Groot MJA, Haagsman HJ, Does MP, van den Elzen PJM, Hooykaas PJJ** (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. *EMBO J* **9**: 3077–3084
- Ow DW** (2002) Recombinase-directed plant transformation for the post-genomic era. *Plant Mol Biol* **48**: 183–200
- Pansegrau W, Schoumacher F, Hohn B, Lanka E** (1993) Site-specific cleavage and joining of single-stranded DNA by virD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proc Natl Acad Sci USA* **90**: 11538–11542
- Peach C, Velten J** (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol Biol* **17**: 49–60
- Rossi L, Hohn B, Tinland B** (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. *Proc Natl Acad Sci USA* **93**: 126–130
- Sambrook J, Fritsch EF, Maniatis T** (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Schlake T, Bode J** (1994) Use of mutated F1p recognition target (f1r) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* **33**: 12746–12751
- Schlam HRM, Hooykaas PJJ** (1997) Effectiveness of the bacterial gene *codA* encoding cytosine deaminase as a negative selectable marker in *Agrobacterium*-mediated plant transformation. *Plant J* **11**: 1377–1385
- Siegel RW, Jain R, Bradbury A** (2001) Using an in vivo phagemid system to identify non-compatible loxP sequences. *FEBS Lett* **505**: 467–473
- Snaith MR, Murray JAH, Boulter CA** (1995) Multiple cloning sites carrying loxP and f1r recognition sites for the Cre and F1p site-specific recombinases. *Gene* **166**: 173–174
- Srivastava V, Anderson OD, Ow DW** (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proc Natl Acad Sci USA* **96**: 11117–11121
- Srivastava V, Ariza-Nieto M, Wilson AJ** (2004) Cre-mediated site-specific gene integration for consistent transgene expression in rice. *Plant Biotechnol J* **2**: 169–179
- Srivastava V, Ow DW** (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. *Plant Mol Biol* **46**: 561–566
- Srivastava V, Ow DW** (2002) Biolistic mediated site-specific integration in rice. *Mol Breed* **8**: 345–350
- Tinland B, Hohn B, Puchta H** (1994) *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus. *Proc Natl Acad Sci USA* **91**: 8000–8004
- Tzvira T, Frankman FR, Vaidya M, Citovsky C** (2003) Site-specific integration of *Agrobacterium* T-DNA via double stranded intermediates. *Plant Physiol* **133**: 1011–1023
- Vergunst AC, De Waal EC, Hooykaas PJJ** (1998a) Root transformation by *Agrobacterium tumefaciens*. In J Martinez-Zapater, J Salinas, eds, *Arabidopsis Protocols*. Humana Press, Totowa, NJ, pp 227–244
- Vergunst AC, Hooykaas PJJ** (1998) Cre/lox-mediated site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* by transient expression of cre. *Plant Mol Biol* **38**: 393–406
- Vergunst AC, Jansen LET, Hooykaas PJJ** (1998b) Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase. *Nucleic Acids Res* **26**: 2729–2734
- Wallace HAC, Marques-Kranc F, Richardson M, Luna-Crespo F, Sharpe JA, Hughes J, Wood WG, Higgs DR, Smith AJH** (2007) Manipulating the mouse genome to engineer precise functional syntenic replacements with human sequence. *Cell* **128**: 197–209
- Zhao X, Coats I, Fu P, Gordon-Kamm B, Lyznik LA** (2003) T-DNA recombination and replication in maize cells. *Plant J* **33**: 149–159
- Ziemenowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L** (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. *Plant Cell* **13**: 369–383
- Zuo JR, Niu QW, Moller SG, Chua NH** (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat Biotechnol* **19**: 157–161
- Zupan JR, Citovsky V, Zambryski P** (1996) *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. *Proc Natl Acad Sci USA* **93**: 2392–2397