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Cre/*lox*-mediated recombination in *Arabidopsis*: evidence for transmission of a translocation and a deletion event

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Abstract. Cre recombinase was used to mediate recombination between a chromosomally introduced *loxP* sequence in *Arabidopsis thaliana* (35S-*lox-cre*) and transferred DNA (T-DNA) originating from *Agrobacterium tumefaciens* (p*lox-npt*), carrying a single *loxP* sequence. Constructs were designed for specific Cre-mediated recombination between the two *lox* sites, resulting in restoration of neomycin phosphotransferase (*nptII*) expression at the target locus. Kanamycin resistant (Km^r) recombinants were obtained with an efficiency of about 1% compared with random integration. Molecular analyses confirmed that these were indeed due to recombination between the *lox* sites of the target and introduced T-DNA. However, polymerase chain reaction analysis revealed that these reflected site-specific integration events only in a minority (4%). The other events were classified as translocations/inversions (71%) or deletions (25%), and were probably caused by site-specific recombination between a randomly integrated T-DNA and the original target locus. We studied some of these events in detail, including a Cre-mediated balanced translocation event, which was characterized by a combination of molecular, genetic and cytogenetic experiments (fluorescence in situ hybridization to spread pollen mother cells at meiotic prophase I). Our data clearly demonstrate that *Agrobacterium*-mediated transfer of a targeting T-DNA with a single *lox* site allows the isolation of multiple chromosomal rearrangements, including translocation and deletion events. Given that the complete sequence of the *Arabidopsis* genome will have been determined shortly this method has significant potential for applications in functional genomics.

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Introduction

Site-specific recombination systems from prokaryotes and yeast, such as the bacteriophage P1 Cre/*lox* system, provide a powerful tool to create precise, controlled genomic rearrangements in higher eukaryotes (Kilby et al. 1993; Van Haaren and Ow 1993; Ow and Medberry 1995; Kühn and Schwenk 1997). Cre recombinase mediates recombination between two 34 bp DNA recognition sequences (*lox*) without requirement for additional factors or any host recombination enzymes. The outcome of a recombination reaction depends on the relative orientation of two *lox* sites towards each other. Intramolecular recombination between two *lox* sites in direct orientation will generate a deletion of intervening DNA sequences, whereas an inverted orientation results in inversion. The presence of two *lox* sites on separate DNA molecules can result in co-integration in the case of circular molecules, or translocation-like events in the case of linear DNA molecules.

In plants, such induced rearrangements have been used for the production of marker-free transgenic plants (Dale and Ow 1991; Gleave et al. 1999), creation of chromosomal deletions or inversions (Medberry et al. 1995; Osborne et al. 1995), isolation of translocations (Qin et al. 1994), reduction of transgene copy number of complex loci (Srivastava et al. 1999), and site-specific integration of transgenes (Albert et al. 1995; Vergunst and Hooykaas 1998; Vergunst et al. 1998a). Furthermore, the possibility of engineering chromosomal rearrangements between preselected sites provides a powerful tool for studying the relation between genomic organization and function, such as the effect of chromosomal position on gene expression (Henikoff 1992; Golic and Golic 1996).

To enable engineering of the plant genome, efficient transformation protocols are required. A widely used method for the production of transgenic plants makes use of the transfer system of *Agrobacterium tumefaciens* (Hooykaas and Beijersbergen 1994; Zupan and Zambryski 1997; Hansen and Chilton 1999). During the *Agrobacterium* infection process a segment of its

tumour-inducing (Ti) plasmid, which is delimited by two short border sequences and called the T-region, is transferred in a single-stranded linear form (T-strand) to the plant cell nucleus where it becomes integrated in the host genome in a random fashion. We have shown previously that the T-DNA from *A. tumefaciens* can be targeted to a previously introduced *lox* sequence in *Arabidopsis* by expressing Cre either transiently (Vergunst and Hooykaas 1998) or at the target locus (Vergunst et al. 1998a), suggesting the possibility of a strategy to eliminate position effects on gene expression (reviewed in Vergunst and Hooykaas 1999). The second approach appeared most successful, resulting in targeted integration with an efficiency of 2% compared with random integration. Since precise site-specific integration in the genome requires circular double-stranded DNA molecules, the single-stranded linear T-strand must have been converted to a suitable substrate for the integration reaction at some stage during the transformation process. Circularization took place, prior to or after random integration, by an initial Cre-mediated recombination event between two *lox* sites that were present on the targeting T-DNA in tandem orientation. Although the number of recombinants that were obtained was very low when Cre was expressed transiently, the use of a T-DNA carrying a single *lox* site resulted in rearrangements other than specific integration, suggesting that circularization by border fusion is a limiting factor for site-specific integration (Vergunst and Hooykaas 1998). More efficient recombination of such a T-DNA might enable the isolation of collections of genomic rearrangements such as translocations or deletions. Here, we describe results of experiments in which a T-DNA containing a single *lox* site was transformed to a Cre-expressing target plant. Selection for site-specific recombination events with the target locus was efficient, and analysis showed that the majority of recombinants were due to deletion, inversion or translocation events. In this paper a detailed characterization of two such events by a combined molecular and cytogenetic analysis is presented.

Materials and methods

Bacterial strains

Strain DH5α of *Escherichia coli* was used for cloning. Standard cloning procedures were used (Sambrook et al. 1989). Recombinant strains were grown in LC medium at 37°C (Hooykaas et al. 1977) supplemented with 25 mg/l kanamycin (Sigma, St. Louis, Mo.) or 100 mg/l carbenicillin (Duchefa, The Netherlands). For plant transformation *Agrobacterium* strain MOG101 (Hood et al. 1993) was used. Bacteria were grown in LC medium supplemented with 100 mg/l kanamycin, 20 mg/l rifampicin and 250 mg/l spectinomycin (Duchefa, The Netherlands) at 29°C. Binary vectors were introduced into MOG101 by electroporation (Mattanovich et al. 1989).

DNA isolation and Southern blot analysis

The isolation of plant chromosomal DNA and Southern blot analysis were performed as described by Vergunst et al. (1998a).

Plasmid constructions

The construction of the p35S-*lox-npt* control vector and the 35S-*lox-cre* target vector was described earlier (Vergunst et al. 1998a). Integration vector p*lox-npt* (see Fig. 1) was constructed

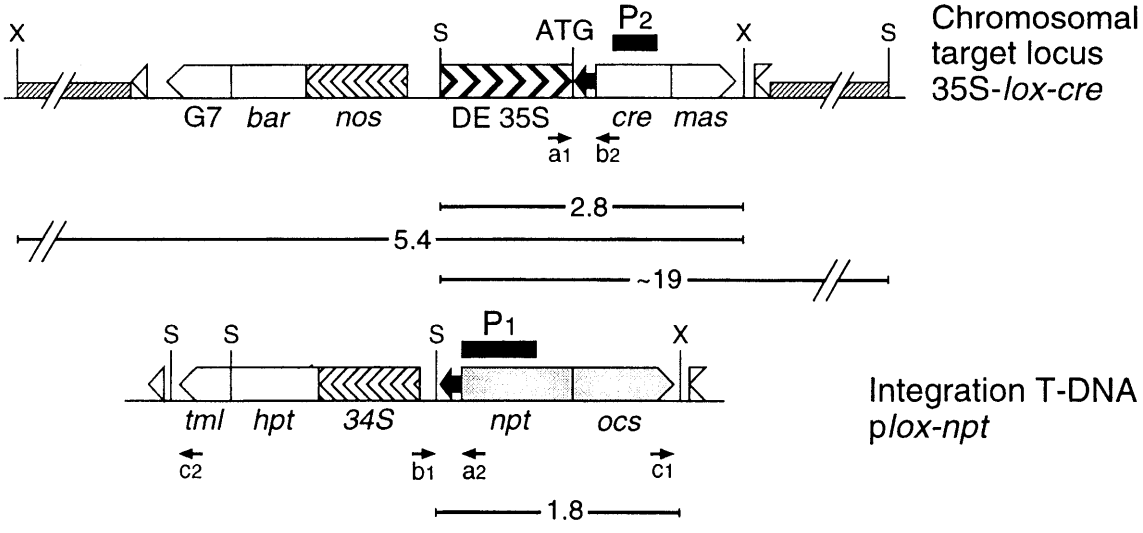


Fig. 1. Schematic representation of the target locus in plant line 35S-*lox-cre* and integration T-DNA p*lox-npt*. *Chevrons* promoter regions; *nos*, *DE35S*, *34S*: promoter regions of the nopaline synthase gene, cauliflower mosaic virus 35S transcript with a double enhancer sequence and AMV leader sequence and 34S transcript of figworth mosaic virus; *bar*, *npt*, *hpt*, *cre*: coding regions of the bialaphos resistance gene, neomycin phosphotransferase gene, hygromycin phosphotransferase gene, and the bacteriophage P1 recombinase gene, respectively; G7, *ocs*, *tml*, *mas*: terminator sequences of *Agrobacterium* gene 7, the octopine synthase gene, tumour morphology large gene, and the mannopine synthase gene, respectively. *Wide black arrow lox* site, *small white triangle Agrobacterium tumefaciens* processed left border repeat, *two small white triangles Agrobacterium* processed right border repeat. *X* XbaI, *S* SacI. *a*, *b* and *c* indicate primer binding sites. Sizes (kb) of hybridizing fragments are indicated. *P1 nptII* probe, *P2 cre* probe

by inserting the *lox-nptII* translational fusion of pSDM3074 (Vergunst and Hooykaas 1998) as a BamHI/NruI fragment in BglII/NruI-digested pIC20R (Marsh et al. 1984), containing the chimeric 34S-*hpt-tml* gene fusion (Vergunst and Hooykaas 1998). A fragment carrying both genes was cloned into binary vector pSDM14 (Offringa 1992).

Polymerase chain reaction (PCR) analysis

Chromosomal DNA from callus or leaf material was isolated essentially according to Lassner et al. (1989). Primers used for the analysis of recombinants were: a1, 5′-GAACTCGCCGTAAAGACTGGCG-3′; a2, 5′-GCGCTGACAGCCGGAACACG-3′; b1, 5′-GGTAATCTTTTTTGTACACTTGCTCG-3′; b2, 5′-CCAGCAGGCGCACCATTGC-3′; c1, 5′-GCGGCAGAACCGGTCAAACC-3′; c2, 5′-GCAAACATCGCCACCCATCG-3′. The reaction mixture (50 µl) contained 50 ng template DNA, 25 pmol of each primer, 200 µM dNTPs and 0.2 U SuperTaq polymerase (HT Biotechnology, England). Amplification by the PCR was carried out in a Perkin Elmer Thermocycler 480, following a standard protocol of 30 cycles: 1 min 95°C, 2 min 57°C and 2 min 72°C.

Plant transformation

The isolation of single-copy target line 35S-*lox-npt* and full details of the transformation experiments were described earlier (Vergunst et al. 1998a). Briefly, T3 or T4 seeds from this line, segregating for the target locus, were used in transformation experiments. Root explants, isolated 10 days after sowing in liquid B5 medium, containing 5 mg/l phosphinothricin (PPT), were incubated for 3 days on callus induction medium (Vergunst et al. 1998b). A 2 day cocultivation period with MOG101(p*lox-npt*) was followed by selection on kanamycin (50 mg/l). The number of kanamycin-resistant (Km^r) calli per root explant was determined 3–5 weeks after cocultivation. Resistant calli were cultured on shoot-inducing medium (Vergunst et al. 1998b) for the production of shoots. After rooting these were transferred to the greenhouse, and allowed to set seed. Genetic analysis was performed by sowing seeds on half-strength MS medium containing 40 mg/l kanamycin or 10 mg/l PPT. Control experiments included transformation of target plant line 35S-*lox-cre* with control strain MOG101(p35S-*lox-npt*) in order to determine the random integration frequency. Cocultivation of wild-type *Arabidopsis* C24 with MOG101(p*lox-npt*) was performed to estimate the frequency of background calli that might arise owing to incidental fusion of the promoterless *nptII* gene to plant regulatory sequences.

Cytogenetic analysis

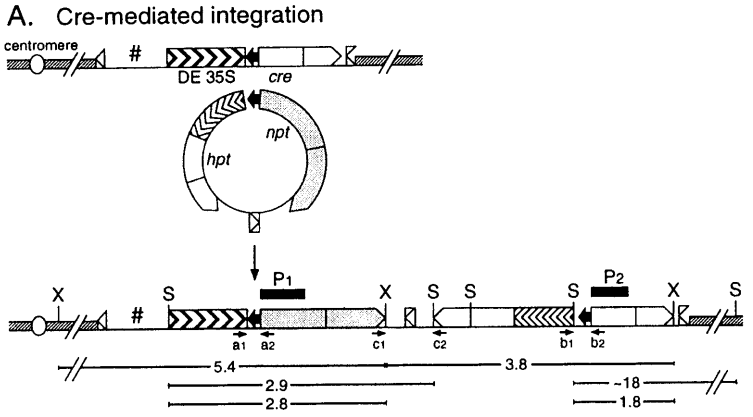
Semisterility was determined by assaying pollen stainability in a few drops of acetocarmine (1.5%) with a trace of ferric chloride and analysis by light microscopy. Flower buds of a T3 plant hemizygous for the recombinant locus R26, were used for producing meiotic prophase I preparations. Tissue fixation, cell spreading and screening for appropriate meiotic stages have been described by Fransz et al. (1998). Selected chromosome preparations were used for fluorescence in situ hybridization (FISH) according to Fransz et al. (1998). The rDNA regions on the chromosomes were hybridized with 5S and 25S rDNA sequences labelled with biotin-dUTP and digoxigenin-dUTP, and detected with rhodamine-labelled streptavidin (red fluorescence) and fluorescein isothiocyanate-labelled anti-digoxigenin (green fluorescence), respectively. The preparations were counterstained and mounted in 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vecta Laboratories). Photomicrographs were taken with a Zeiss Axiophot Photomicroscope on 400 ISO colour negative films and scanned at 1000 dpi for digital processing and reproduction.

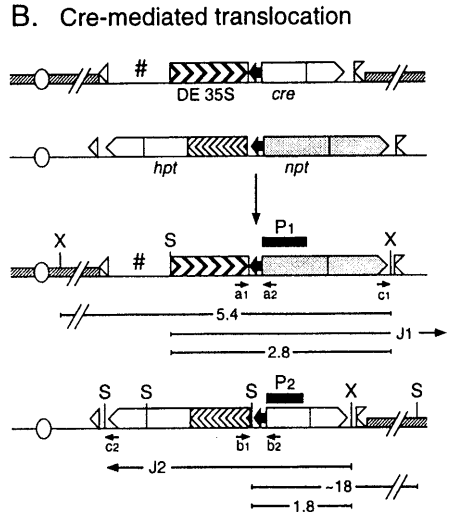
Results

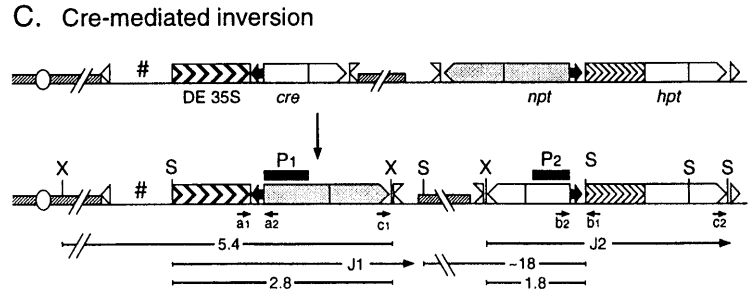
Experimental design

Selection for site-specific recombination events between an incoming T-DNA and a previously introduced *lox* sequence in the plant genome was achieved by directing a promoterless *nptII* gene to the missing transcriptional signals present at the chromosomal target site. More specifically, a target plant (35S-*lox-cre*) containing a single copy of the *cre* open reading frame translationally fused to a *lox* site and driven by a strong promoter sequence, was isolated using the Basta resistance gene as a selectable marker (Vergunst et al. 1998a; Fig. 1). The targeting T-DNA used for recombination experiments contained a single *lox* sequence translationally fused to a promoterless *nptII* gene, which in addition lacked the ATG start codon (Fig. 1). Following transformation of the T-DNA to target plant cells, Cre-mediated recombination events were selected by activation of the *nptII* gene upon capturing the ATG start codon and promoter sequence of the *cre* gene. At the same time resolution of Cre-mediated recombination products was prevented by the disconnection of the *cre* open reading frame from these signals (Qin et al. 1994; Vergunst et al. 1998a).

Depending on the fate of the linear single-stranded T-DNA on entering the plant cell nucleus, several recombination events can be envisaged (Fig. 2) that can lead to restoration of *nptII* expression. These events are: (1) targeted integration of a circular double-stranded DNA molecule (Fig. 2a). Such a circular substrate may be formed by extrachromosomal circularization (border fusion) and second strand formation of T-DNA p*lox-npt*, but also after initial random integration of p*lox-npt* in a directly repeated structure, which creates a substrate for Cre-mediated excision at the then directly oriented *lox* sites within the repeat. (2) Initial random integration of p*lox-npt* T-DNA followed by recombination with the 35S-*lox-cre* target locus. Depending on the position and orientation of the T-DNA with respect to the genomic target *lox* site the following recombination events can be envisaged: firstly, if the p*lox-npt* T-DNA integrated in a chromosome different from the chromosome containing the target *lox* site, a translocation may arise (Fig. 2b). Balanced translocation events will result if both *lox* sites are in the same orientation with respect to the centromeres, leading to a reciprocal exchange of chromosomal arms. Acentric and dicentric chromosomes, which result from recombination between *lox* sites of opposite orientation with respect to the centromere, are most likely lethal for the cell. Secondly, random integration on the chromosome at which the target *lox* site is located can result in inversion events (Fig. 2c; random integration, positioning the *lox* sites in opposite orientation) or deletion events (Fig. 2d; random integration at the right side of the target locus, positioning the *lox* sites in direct orientation). Events taking place at the left side of the target locus will not be detected in this case owing to deletion of the *nptII* gene. (3) In theory, Cre-mediated recombination of a linear T-DNA with the target site will lead to chromosomal breakage. However, it can be en-







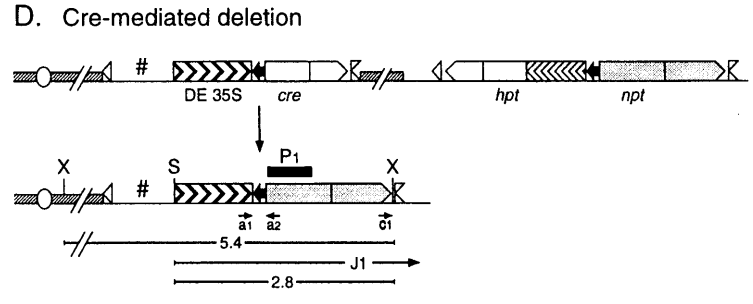


Fig. 2A–D. Schematic representation of Cre-mediated recombination events that can lead to a Km^r phenotype after introduction of T-DNA p*lox-npt* into target plant line 35S-*lox-cre*, and the molecular analysis. The *bar* gene is not drawn (see Fig. 1) for clarity. The position of the centromere is arbitrary. **A** After circularization of the T-DNA (p*lox-npt*) Cre-mediated recombination can lead to site-specific integration. A border fusion will then be present at the recombinant locus. **B** Random integration of p*lox-npt* on a chromosome other than that on which the target locus resides, and positioning of the *lox* sites in the same orientation with respect to the centromere, can lead to Cre-mediated reciprocal exchange of chromosomal arms, resulting in a balanced translocation. **C** Random integration of p*lox-npt* at a position linked to the target locus, with the *lox* sites in opposing orientation, can lead to Cre-mediated inversion events. **D** Deletion events can result after random integration of p*lox-npt* at a position linked to the target locus, positioning the *lox* sites in direct orientation. *Wide black arrow* *lox* site, *small white triangle* *Agrobacterium tumefaciens* processed left border repeat, *two small white triangles* *Agrobacterium* processed right border repeat, *white triangle in box* fused borders. *a*, *b* and *c*, positions of primer binding sites. *P1 nptII* probe, *P2 cre* probe. *S* SacI, *X* XbaI. Sizes of restriction fragments (kb) are indicated. *J1* and *J2*, new border fragments. *Hash* represents the *bar* gene (see Fig. 1). The panels are not drawn to scale

Table 1. Transformation efficiency of target line *35S-lox-cre* with integration vector p*lox-npt*

Experiment	Random integration frequency[a]	Number of explants	Number of Km^r calli[b]	Frequency of Km^r calli[c]	Ratio[d]
1	2	2160	56	2.6×10^{-2}	1:81
2	2	360	12	3.3×10^{-2}	1:60
3	1	535	4	0.7×10^{-2}	1:134

[a] Number of Km^r calli per root explant after transformation with control vector p35S-*lox-npt*
[b] Number of Km^r calli that survived selection in recombination experiments. Fourteen calli that were initially green but died in a later stage were not included
[c] Number of Km^r calli per root explant in recombination experiments
[d] Ratio of Km^r calli obtained after transformation in recombination experiments (c) and those obtained after transformation with the control vector (a)

Table 2. Classification of isolated recombinants based on polymerase chain reaction (PCR) analysis. Class I indicates precise integration (positive with primer combinations a, b and c), class II contains translocation and/or inversion events (positive with primer combination a and b), while Class III consists of deletion events (positive with primer combination a only). The percentage of regenerating calli, shoots, and the percentage of calli resulting in fertile offspring are indicated

Exp.	No. Km^r calli	No. (%) in PCR class			Regeneration (%)			Seed set (%)		
		I	II	III	I	II	III	I	II	III
1	56	3 (5)	36 (65)	17 (30)	100	89	82	33	53	53
2	12		12 (100)			83			67	
3	4		3 (75)	1 (25)		33	100		33	100

visaged that simultaneous illegitimate recombination of the right T-DNA end with plant DNA (end-joining) may result in a deletion event, similar to the one depicted in Fig. 2d.

Transformation experiments

A mixture of root explants, either hemizygous or homozygous for target locus 35S-*lox-cre*, were cocultivated with *Agrobacterium* strain MOG101(p*lox-npt*). It was shown earlier that this plant line expresses the Cre recombinase (Vergunst et al. 1998a). In three independent recombination experiments, selection resulted in the isolation of 72 Km^r calli (see Table 1). The random integration frequency, estimated with control vector p35S-*lox-npt*, was between 1 and 2 calli per root explant, whereas the frequency of Km^r calli in recombination plates varied from 0.7×10^{-2} to 3.3×10^{-2} calli per explant. The ratio of calli isolated in recombination experiments and those obtained with the control vector thus varied from 1 in 60 (1.7%) to 1 in 134 (0.7%). In control experiments *Arabidopsis* C24 wild-type roots were cocultivated with MOG101 (p*lox-npt*). No Km^r calli were obtained in these experiments. The isolation of Km^r calli in recombination experiments was therefore likely caused by Cre-mediated recombination between the *lox* site residing on the T-DNA and the target *lox* site, restoring *nptII* expression.

Classification of the recombinants by PCR analysis

To distinguish between site-specific integration, deletion and translocation or inversion events, PCR analysis was performed. This analysis was based on the presence of the new border junctions that will result after Cre-mediated recombination between the target and introduced T-DNA sequences. A site-specific integration event will lead to amplification of specific fragments with the three primer sets a, b and c (Fig. 2a). Primer combination c is indicative of a fusion between the left and right border sequences of the T-DNA, which should be present in such precise integrants at the target locus. However, a randomly integrated tandem repeat structure also contains a left/right border fusion (primer combination c). This requires further analysis with primer combinations c2 and a1, as well as c1 and b2, to confirm site-specific integration. If amplification is detected with primer combinations a and b, but not with c, this is indicative of translocation or inversion events (Fig. 2b and 2c). A Cre-mediated deletion event will result in amplification of a specific fragment with primer set a only (Fig. 2d).

In this way 72 Km^r calli, isolated from recombination experiments were analysed. Three resistant calli (4% calculated from three experiments), of which only one yielded fertile offspring (R21), gave amplification of the fragments predicted for precise integration (Class I, Table 2) with primer sets a (0.7 kb), b (1.1 kb) and c (0.6 kb; see R21 in Fig. 3). Amplification of the predicted fragments with primer combination c2 and a1, as well as c1 and b2 (Fig. 2a; data not shown) confirmed that the complete T-DNA (p*lox-npt*) was indeed linked to the target locus. Three other resistant calli also yielded a positive signal with primer sets a, b and c, but not with c2-a1 and c1-b2 (Class II, Table 2). The positive signal with c in these recombinants therefore suggests that a tandem repeat structure was present besides a translocation/inversion event. DNA samples from another 48 calli gave amplification with primer combinations a and b, which is indicative of translocation or inversion events (Class

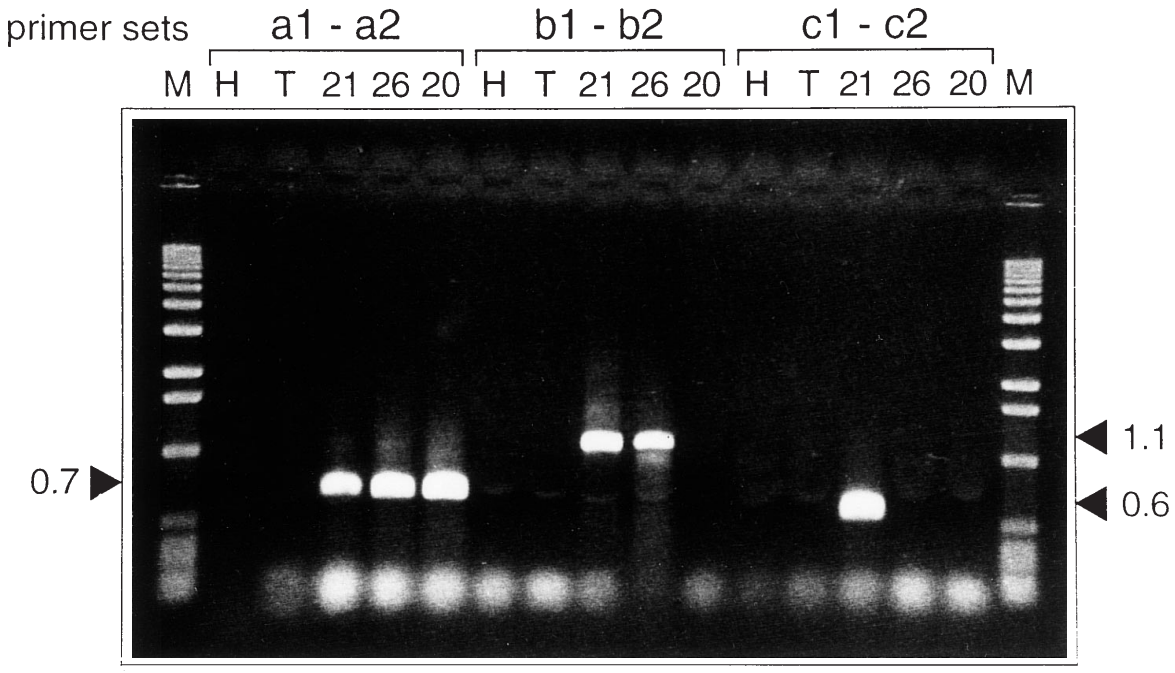


Fig. 3. Ethidium bromide-stained agarose gel from polymerase chain reaction analysis of three Km^r calli, representing class I (*R21*), II (*R26*) and III (*R20*). *T* target line 35S-*lox-cre*, *M* molecular weight marker, *H* water control. Primer binding sites are indicated in Figs. 1, 2

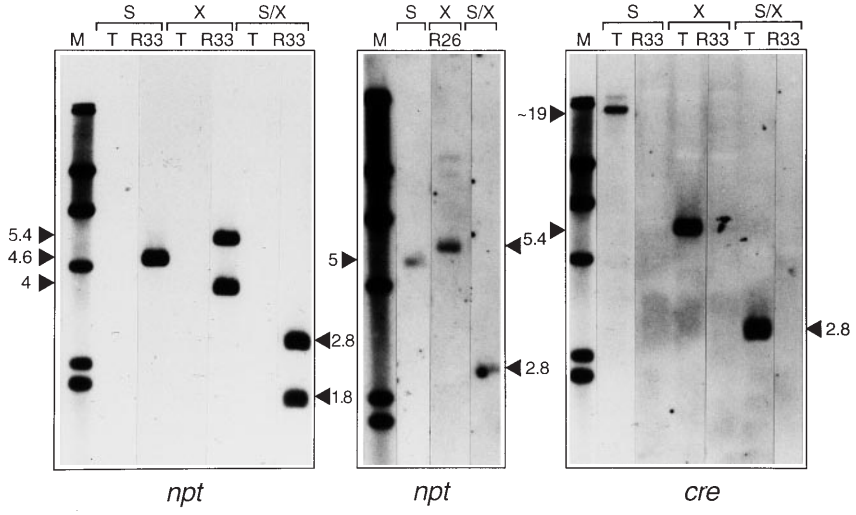


Fig. 4. Southern blot analysis of target line 35S-*lox-cre* (*T*), R26 (Class II) and R33 (Class III). Indicated are sizes (kb) of fragments (Figs. 1, 2) after hybridization with *npt* or *cre* probes. *X* XbaI, *S* SacI, *M* digoxygenin-labelled marker

II in Table 2; shown for recombinant R26 in Fig. 3). The remaining 25% of the Km^r calli gave a positive signal only with primer combination a, confirming that precise recombination at the *lox* sites, restoring *nptII* expression, had occurred. In contrast to the other recombinants, no right *lox* junction (b) could be detected, which indicates deletion on this side (Class III in Table 2; R20 in Fig. 3). This was confirmed in five of the recombinants by the absence of the *cre* gene as indicated by PCR analysis (data not shown). In DNA samples from the other recombinants carrying a deletion, a fragment was amplified with *cre* primers. Probably these recombinants resulted after deletion of only one allele in cells that were originally homozygous for the target locus; in DNA samples from these recombinants original target locus fragments were amplified as well. It cannot be excluded, however, that some root material from the original target line was present during DNA isolation, resulting in a positive signal with *cre* primers. Table 2 summarizes the classification of the isolated recombinants. In addition the percentage of resistant calli that generated shoots and yielded fertile offspring is given.

Southern blot analysis

To obtain more conclusive evidence about the nature of the recombination events a number of the recombinants were subjected to Southern blot analysis in the T2 generation. As shown in Fig. 4, DNA from target line

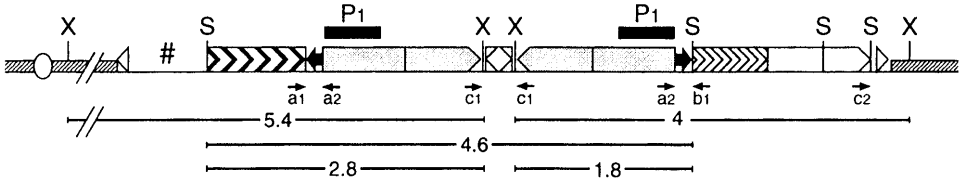


Fig. 5. Schematic representation of the deletion event in R33 (Class III), as deduced from Southern blot analysis. For explanation see legends to Figs. 1, 2

35S-*lox-cre* (T) yielded fragments of about 19 kb (SacI), 5.4 kb (XbaI) and 2.8 kb (SacI/XbaI), respectively (indicated in Fig. 1) after hybridization with a *cre* probe (P2). As expected no hybridization with a *nptII* probe (P1) occurred. Cre-mediated recombination of T-DNA p*lox-npt* with the target *lox* site will then result in hybridization of 5.4 kb XbaI and 2.8 kb SacI/XbaI fragments with a *nptII* probe (see Fig. 2a–d). Furthermore, a 2.9 kb SacI fragment will also be present in precise integrants (Fig. 2a; data not shown), whereas in the case of translocation, inversion or deletion events new SacI fragments (indicated as J1 in Fig. 2b–d will hybridize with a *nptII* probe.

In recombinants R26 and R33, which were designated by PCR analysis as translocation or inversion (class II) and deletion events (class III), respectively, the expected 5.4 kb XbaI and 2.8 kb SacI/XbaI fragments hybridizing to a *nptII* probe, indicative of Cre-mediated recombination, were detected (Fig. 4). However, new junctions, visualized by a 5 kb SacI fragment for R26 and an about 4.6 kb SacI fragment for R33 (*nptII* probe) indicated a physical separation of both recombinant junctions, corroborating PCR data. Furthermore, the absence of hybridization with a *cre* probe in R33 confirmed that a deletion event had occurred, leading to complete loss of the *cre* gene. The hybridization of an additional fragment in DNA isolated from this recombinant with a *nptII* probe in the XbaI (4 kb) and the SacI/XbaI double digest (1.8 kb), but not in the SacI digest, suggests the structure presented in Fig. 5. The fact that a single hybridizing fragment was detected in the SacI digest in contrast with the double digest indicated a physical linkage of two integration T-DNAs in an inverted orientation. Furthermore, the absence of additional fragments hybridizing with a *nptII* probe showed that no extra illegitimate recombination events had taken place in those recombinants. In conclusion, Southern hybridization data support the PCR results showing that in R26 a Cre-mediated inversion or translocation had occurred, whereas in R33 a deletion event between an inverted repeat and the target locus had occurred.

Cytogenetic characterization of a Cre-mediated translocation event

In order to discriminate between Cre-mediated translocation and inversion events, which was not possible on the basis of molecular analysis alone, we decided to analyse line R26 (Class II) cytogenetically. In the case of a translocation heterozygote, the two non-translocation and two (reciprocal) translocation chromosomes of the translocation complex can pair at pachytene. If crossovers occur in the four pairing arms, a quadrivalent appears at metaphase I. Depending on the orientation of adjacent centromeres, such a quadrivalent may display alternate orientation with the two translocation chromosomes facing one pole and the non-translocation chromosomes facing the opposite pole. Segregation of the chromosomes at anaphase I ultimately gives rise to four balanced gametes, two with and two without the translocation. Alternatively, in the case of adjacent orientation of the four chromosomes of the translocation complex, a non-translocated and a translocated chromosome face the same pole, and unbalanced gametes containing a combination of chromosomal duplication or deficiency are formed. If both adjacent and alternate orientations occur, semisterility will arise. Heterozygous plants of R26 indeed showed reduced fertility and pollen viability. The segregation ratio for kanamycin resistance (representing a possible translocation breakpoint) of an individual T2 plant was 78:11. Thirty-seven of the 78 resistant plants displayed the phenotype shown in Fig. 6. A T3 plant constituting this phenotype segregated 71:15 for Km^r in its offspring, whereas a T3 plant of R26 displaying a wild-type phenotype segregated 77:36. This plant did not segregate for plants with a visible phenotype.

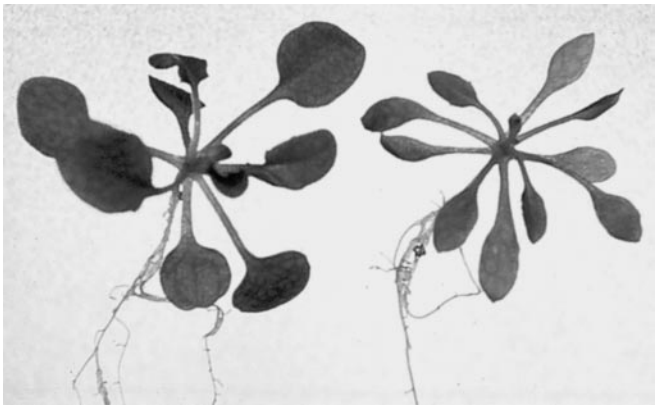


Fig. 6. Phenotype of R26 (*right*) and a wild-type plant

Meiotic metaphase I preparations of spread pollen mother cells were made from flower buds of a plant that was heterozygous for the recombinant locus (Km^r) as shown by segregation analysis in the offspring. In addition, this plant displayed the phenotype mentioned earlier. It was anticipated that a plant that segregated in its offspring for kanamycin resistance (representing the breakpoint) would be heterozygous for the translocation. Microscopic analysis of metaphase I complements of R26 revealed the expected translocation quadrivalents. In 22 out of 25 metaphases, multivalents were clearly

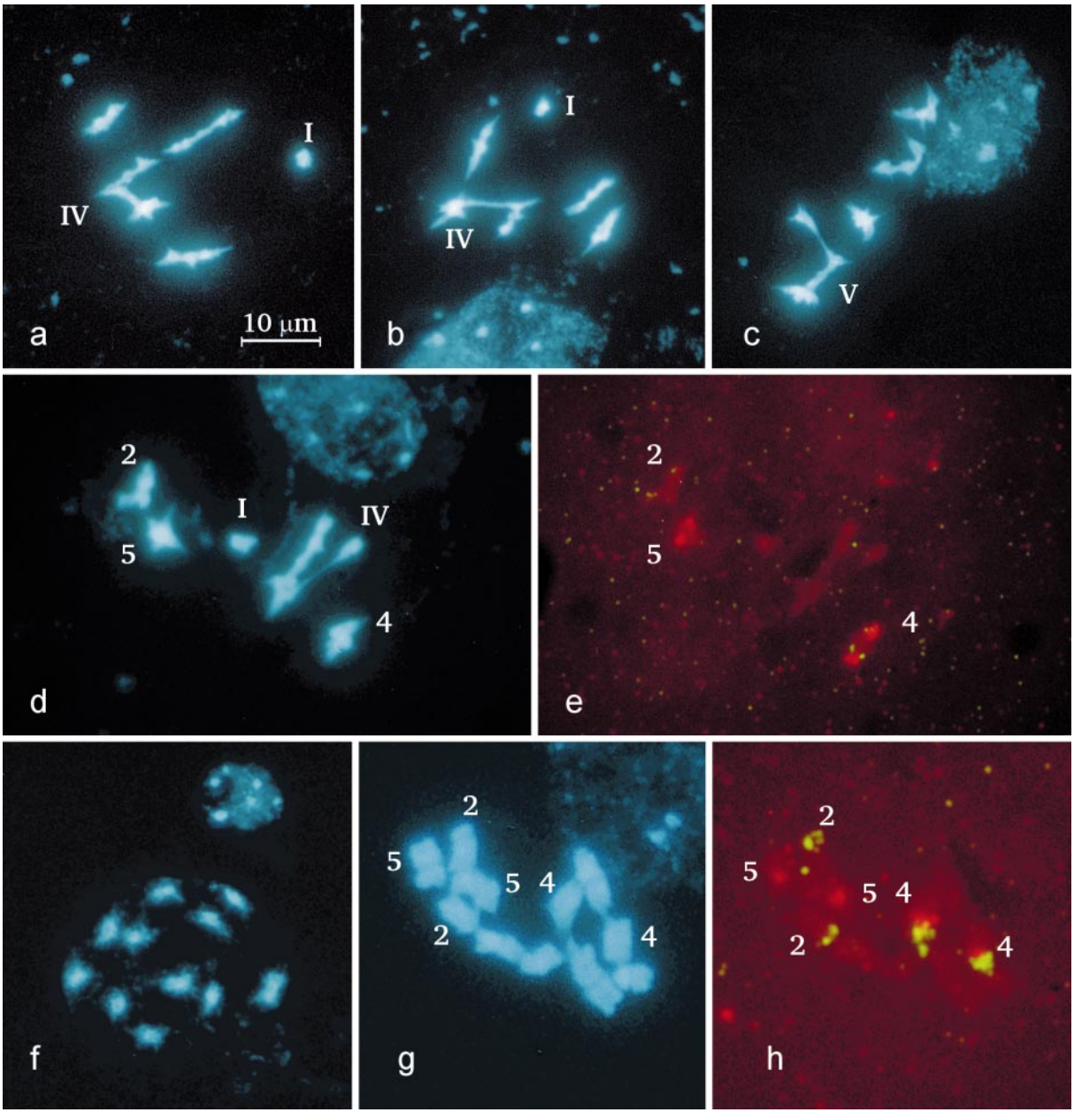


Fig. 7. a,b,d 4′,6-Diamidino-2-phenylindole (DAPI)-stained metaphase I spreads of R26 showing three bivalents, a quadrivalent (*IV*) and a univalent (*I*). **c** DAPI-stained metaphase I spread of R26 with a possible quinquevalent (*V*). **e** Fluorescence in situ hybridization (FISH) image of R26 metaphase I (**d**), hybridized with 25S rDNA (*green signal*, chromosomes 2 and 4) and 5S rDNA (*red signal*, chromosomes 4 and 5) probes. **f** DAPI-stained tapetal cell in mitotic metaphase. **g** DAPI-stained mitotic metaphase of R26. **h** FISH image of **g**, hybridized with 25S rDNA (*green signal*) and 5S rDNA (*red signal*) probes

observed (see Fig. 7a–d). Eight of them showed the quadrivalent and the three bivalents, plus an additional univalent (Fig. 7a, b, d), which was also observed in mitotic metaphase cells (Fig. 7f, g). In the remaining 14 cells the extra chromosome was involved in the translocation complex, forming a pairing configuration of five chromosomes (quinquevalents; Fig. 7c). These data indicated that R26 contained an additional chromosome that was (partly) homologous to one of the chromosomes of the translocation.

As DAPI-stained metaphase chromosomes cannot be identified morphologically, we applied FISH with 5S and 25S rDNA as probes. In *Arabidopsis* C24 the 5S rDNA probe hybridizes to chromosomes 4 and 5 (red signal), whereas the 25S rDNA probe hybridizes to chromosomes 2 and 4 (green signal). The multivalents, as unequivocally observed in 10 out of 17 metaphase I cells, did not display either FISH signal (Fig. 7e), indicating that the chromosomes involved in the translocation were chromosomes 1 and 3. Owing to the faintness of the green fluorescent signal of the 25S rDNA it was difficult to distinguish chromosomes 2 and 4 in the other seven metaphases, although a clear red fluorescence confirmed the absence of chromosomes 4 and 5 in the

translocation complex in four metaphase I spreads. We never observed hybridization of the probes with the univalent. Fluorescence in situ hybridization of a mitotic metaphase (Fig. 7h) shows that two homologs are present of each of chromosomes 2 (green signal), 4 (green and red signal) and 5 (red signal). Five non-hybridizing chromosomes therefore indicate the presence of an additional chromosome 1, chromosome 3, a translocation chromosome 1 or a translocation chromosome 3.

Discussion

Recombination efficiency

Here, we describe the isolation and detailed analysis of Cre-mediated translocation and deletion events after recombination of a T-DNA vector harbouring a single *lox* site with a previously introduced *lox* site in the *Arabidopsis* genome. Correction of a defective kanamycin resistance gene resulted in the isolation of Km^r calli with an efficiency of about 1% compared with random integration. Polymerase chain reaction analysis showed that the kanamycin resistance was indeed the result of physical linkage of the promoterless *nptII* gene from the introduced T-DNA to the cauliflower mosaic virus (CaMV) 35S promoter region present at the target *lox* site. Molecular analysis indicated that only 4% of the resistant calli resulted from targeted integration at the target locus. The remaining 96% resulted from Cre-mediated recombination events between the target *lox* site and the *lox* site of a randomly integrated T-DNA. Of these, 25% were probably the result of deletion events, whereas 71% were likely caused by translocation or inversion events.

Deletion events

Chromosomal deletions will be helpful for the identification and cloning of genes of which only the mutant phenotype is known. Van Haaren and Ow (1993) described a strategy to create genomic deletions by the combined use of transposition and site-specific recombination [transposition-deletion (TRADE) system]. By using the Cre/*lox* system and the "two-element" *Ac/Ds* transposition system, Medberry et al. (1995) and Osborne et al. (1995) found evidence for genomic inversions and a deletion event. Our data suggest an efficient and less laborious way to isolate genomic deletions, namely by transformation of a target plant harbouring a genomic *lox* site with a single-*lox* T-DNA. A rapid screen of the transformed population would allow selection of those recombinants that have lost the *cre* gene. Southern blot analysis revealed a Cre-mediated deletion in line R33 due to recombination between the target *lox* site and a *lox* site of a T-DNA repeat structure (see Fig. 5). Segregation for the Km^r phenotype in a 2:1 ratio suggested that the deletion was lethal in the homozygous condition, but confirmed transmission of the deletion to the next generation. It would be very interesting to determine the size of the chromosomal deletion and the genes affected by the deletion in R33, as the haploid gametophyte seemed unaffected by the loss of gene function.

Deletions, produced by the method described in this paper, can be due to integration of the T-DNA at a position linked to the target locus, positioning its *lox* site in a direct orientation with the target *lox* site, followed by Cre-mediated deletion. Alternatively, repair of chromosomal breaks induced by Cre-mediated recombination between the target *lox* site and the *lox* site of a linear extrachromosomal T-DNA may lead to deletion-like events, similar to those found after gene targeting by homologous recombination. Such one-sided recombination events, in which one end of the T-DNA has integrated via homologous recombination, while the other side has integrated by illegitimate recombination (Risseeuw et al. 1995; Puchta 1998), have been observed frequently in plant gene targeting experiments.

Translocation events

Qin et al. (1994) described the use of the Cre-*lox* system to induce chromosomal translocations in plants. Separate plant lines were constructed, each harbouring a copy of either of two *lox* constructs. After crossing individual plants, the two *lox* loci and the *cre* gene were combined into one genome. Expression of Cre led to recombination events, detected by reconstruction of a selectable marker gene. However, only in 3 out of 16 combinations were translocation events detected. Furthermore, analysis for the occurrence of recombination events at several time points after fertilization showed that in time saturation occurred. The data presented in this paper demonstrate an alternative way to isolate translocation events. Only a single-copy target plant is required to select for translocation events between a specific genomic location and genomic positions determined by the random integration site of the T-DNA. A fast PCR screen in the callus stage would allow the isolation of inversion or translocation events, without additional random events, followed by identification with sensitive in situ hybridization.

Fluorescence in situ hybridization provides a powerful technique for molecular cytogenetic analysis in *Arabidopsis* and other higher plants (reviewed in De Jong et al. 1999). Recently, Fransz et al. (1998) used this technique to present a detailed karyotypic analysis of several *Arabidopsis* ecotypes using meiotic chromosomes. In this paper, cytogenetic analysis, including FISH to spread pollen mother cells at meiotic prophase I, allowed us to extend our observation from molecular analysis that a translocation event had occurred in R26. Combined molecular, genetic and cytogenetic analysis provided strong evidence for a Cre-mediated translocation event between two *lox* sites on chromosomes 1 and 3, reconstituting a kanamycin resistance marker. The additional chromosome, seen in the metaphases of a heterozygous R26 plant (T3), which also showed a characteristic phenotype (Fig. 6), did not hybridize to either 5S or 25S rDNA probes, indicating that the chromosome in-

volved is likely normal chromosome 1 or 3, a translocation chromosome 1 or 3, or any other chromosomal rearrangement not possessing the diagnostic rDNA sites. The observed phenotype was described earlier (Koornneef 1994) for plants trisomic for chromosome 3, but not for plants trisomic for chromosome 1. This suggests that the additional chromosome is chromosome 3 or the translocated chromosome 3. The origin of the additional chromosome can easily be explained from irregular segregation of chromosomes during meiosis (Sybenga 1992). In the translocation heterozygote (T1), several pairing configurations during meiosis may have resulted in the formation of balanced gametes with an additional chromosome. In these cases a normal complement (chromosomes 1 and 3) will have an additional translocated chromosome 1 or 3; a translocation complement will have an additional normal chromosome 1 or 3. Combination of a gamete with an additional chromosome and a normal gamete will result in a translocation trisomic. Assuming that transmission of the trisomic through the male germ line does not occur (Koornneef and van der Veen 1983), and that chromosomal deficiencies are also not transmitted, trisomic segregation in the translocation heterozygote will result in strong deficits of the double recessives, in the case where the additional chromosome is a translocation chromosome. This is in agreement with our data for segregation on kanamycin in the offspring of a phenotypically altered plant (78:11; 71:15; $P>0.05$), and contrasts with the situation where the translocation chromosome is not the additional one.

The use of Cre/*lox* to induce translocations provides a clean system. Previous studies, using X-rays and fission neutrons for the induction of translocations, revealed high background induced damage (Sree Ramulu and Sybenga 1979, 1985). A combined use of homologous recombination and site-specific integration as described in mammalian cells may allow very powerful studies of the effect of programmed translocations (Smith et al. 1995; Van Deursen et al. 1995) in the future.

Site-specific integration

Our results indicate that a T-DNA with a single *lox* sequence is not efficiently converted into a suitable double-stranded circular DNA molecule for precise site-specific integration. This may be due to a low frequency, or even the absence, of extrachromosomal circularization, and/or second-strand synthesis of the single-stranded T-DNA. However, the isolation of three site-specific integrants (0.05% of random integration events, i.e. 4% of the Km^r calli) in this study must have resulted from site-specific integration of such circular intermediates. In vitro experiments (Pansegrau et al. 1993), as well as T-DNA transfer experiments in yeast (Bundock et al. 1995) and agro-infection in turnip (Bakkeren et al. 1989) have shown that circularization of T-DNA can occur. Formation of such T-circles in the current experiments may well have resulted from excision of a randomly integrated head-to-tail direct-repeat structure, rather than being the result of extrachromosomal circularization through border fusion. Such a configuration would position two *lox* sites in a direct orientation, a good substrate for Cre-mediated excision and formation of a double-stranded circular DNA molecule. Earlier we suggested (Vergunst and Hooykaas 1998) that border fusion of a single-*lox* T-DNA designed for site-specific integration was inefficient and that such a vector should therefore contain two *lox* sites to allow Cre-mediated circularization. In those experiments the number of recombinants obtained was very low, probably owing to the method of expressing the Cre recombinase transiently. By expressing Cre in the target plant we showed that a T-DNA containing two *lox* sites indeed yielded site-specific integrants with an efficiency of 2% compared with random integration (Vergunst et al. 1998a). Expressing Cre this way also increased the number of recombinants obtained after transformation of a single *lox* T-DNA as shown in this paper, and allowed a detailed analysis of the types of recombination event that had occurred. The results clearly show that the choice of the *Agrobacterium* T-DNA vector determines the outcome of the recombination events, and allows the isolation of diverse genomic rearrangements in plants. Given that the complete genomic sequence of *Arabidopsis* will be available soon, determination of DNA sequences flanking the target locus and T-DNA integration site will allow immediate classification of inversions, translocations and the size of deletions, providing an additional tool in functional genomics.

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