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Genetics

Nonreciprocal homologous recombination between *Agrobacterium* transferred DNA and a plant chromosomal locus

(*Nicotiana tabacum*/protoplasts/gene targeting/gene conversion/neomycin phosphotransferase II)

REMKO OFFRINGA*†, MARRY E. I. FRANKE-VAN DIJK*, MARCEL J. A. DE GROOT‡, PETER J. M. VAN DEN ELZEN‡, AND PAUL J. J. HOOYKAAS*

*Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands; and ‡Mogen International nv, Einsteinweg 97, 2333 CB Leiden, The Netherlands

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ABSTRACT **Previously, we demonstrated the occurrence of gene targeting in tobacco cells after *Agrobacterium*-mediated transformation. In these experiments a defective kanamycin resistance (Km^r) gene residing at a chromosomal location was restored via homologous recombination with an incoming transferred DNA (T-DNA) repair construct (pSDM101) containing a different defective Km^r gene. In this article we describe gene targeting experiments with the same target line, but using an improved repair construct, pSDM321. In one of the Km^r calli obtained after transformation with pSDM321 (line A) the product of homologous recombination was detected using PCR. Further molecular analysis revealed that the defective Km^r gene present on the incoming T-DNA had been restored via homologous recombination with the target locus. The target locus was left unchanged and the corrected T-DNA was found to be inserted on the same chromosome but not close to the target locus. This paper presents molecular evidence in plants for the conversion of an introduced DNA molecule (in this case, T-DNA) by a homologous chromosomal locus.**

The integration of foreign DNA in higher eukaryotic cells occurs at random loci through a process that is referred to as illegitimate recombination (1–3). Gene targeting, defined as the integration of introduced DNA via homologous recombination into the genome, occurs only at a relatively low frequency. Nonetheless, it has become a well-established tool for the specific inactivation or modification of genes in some mammalian systems (4). Paszkowski *et al.* (5) were the first to report that homologous recombination between a target locus and an incoming purified DNA molecule does occur in plant cells, albeit at a very low frequency.

In previous experiments we investigated the potential use of *Agrobacterium* transferred DNA (T-DNA) for gene targeting in plants. The transgenic tobacco line T, which is hemizygous for a T-DNA with a defective kanamycin resistance (Km^r) gene (target locus), was retransformed via *Agrobacterium* with a T-DNA containing a defective Km^r gene with a complementing nonoverlapping mutation (repair construct). Among 213 kanamycin-resistant calli selected from a total of 10^5 transformants, one recombinant line was identified by PCR analysis. In this line the defective Km^r gene at the target locus had been properly restored (6).

To study the process of *Agrobacterium*-mediated gene targeting in more detail we attempted to reduce background events and to increase the detection sensitivity for homologous recombination events. Two important modifications were made to the original repair construct pSDM101: (*i*) the *aux-2* gene was inserted as a segment of nonhomologous DNA between the right T-DNA border and the promoterless defective neomycin phosphotransferase II (NPTII) gene to reduce the formation of gene fusions after integration of the repair T-DNA; (*ii*) a 137-bp deletion was introduced into the 3′ noncoding region of the defective Km^r gene in the repair construct, thus allowing clear distinction between a product of homologous recombination and the wild-type construct.

Protoplasts of plant line T, which had been used in the previous experiments (6), were cocultivated with an *Agrobacterium* carrying the modified T-DNA. From these experiments a recombinant line (A) was isolated, of which a detailed molecular analysis is presented here.

MATERIALS AND METHODS

Constructs. The binary vectors were derived from plasmids pSDM100 and pSDM101 (6) using standard molecular techniques (7). A base-pair substitution in the NPTII coding region (8) was corrected in pSDM100 and pSDM101 (reintroduces an *Xho* II site). In this way pSDM300 (not shown) was derived from pSDM100. Following this base-pair exchange in pSDM101, the 137-bp *Sma* I/*Pst* I fragment was deleted from the 3′ noncoding region of the Km^r gene, which resulted in pSDM301 (not shown). A 2543-bp *Hin*dIII partial that contained the *aux-2* gene and the 5′ part of the *aux-1* gene of pTiAch5 (positions 3390–5933) (9) was cloned into the *Hin*dIII site of pIC20R (10). The 5′ part of *aux-1* was removed up to the *Hin*cII site at position 5721 (9) by digestion with *Hin*cII and *Pst* I (one of the multiple cloning sites of pIC20R) and religation. Subsequently, the *Xho* I and *Sal* I sites of pIC20R were removed by digestion with these enzymes followed by ligation of the fragment in inverse orientation. Finally, the *aux-2* gene was cloned as a *Bam*HI/*Bgl* II partial at the *Bcl* I site of pSDM300 or as a *Bam*HI/*Eco*RI partial at the *Eco*RI site of pSDM301. The resulting plasmids are referred to as pSDM320 and pSDM321, respectively (see Fig. 1). The plasmids were mobilized (11) into *Agrobacterium* strain GV2260 (12) to form strains SDM320 and SDM321, respectively.

Plant Tissue Culture. The method for cocultivation of tobacco protoplasts (*Nicotiana tabacum* Petit Havana line SR1), the origin of tobacco plant line T (originally referred to as line 104), and plant tissue culture media were described previously (6).

PCR, Inverse PCR, and Sequence Analysis. Plant DNA for PCR analysis was isolated according to Lassner *et al.* (13). PCR was performed in a Perkin-Elmer thermocycler 480 using a standard protocol of 30 cycles: 1 min, 95°C denaturation; 1 min, 57°C annealing; 2 min, 72°C elongation. The

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Abbreviations: Km^r/Hm^r, kanamycin/hygromycin resistance; NPTII, neomycin phosphotransferase II; DSBR: double-strand break repair; T-DNA, transferred DNA; HPT, hygromycin phosphotransferase; NOS, nopaline synthase.

†To whom reprint requests should be addressed.

reaction mixture contained 0.1 unit *Taq* DNA polymerase (HT Biotechnology, Cambridge, U.K.), 100 μM dNTPs (Amersham), and 25 pmol of each primer.

Inverse PCR was performed as described (14) except that restriction endonucleases *Hpa* II and *Sac* II and primers 7 and 8 were used (see Fig. 4*A*). Amplification occurred in a 35-cycle reaction and annealing at 58°C. Sequence analysis was performed using the Sequenase 2.0 DNA sequencing kit (United States Biochemical).

DNA Isolation and Southern Analysis. Isolation of plant DNA and Southern analysis were performed essentially as described (6). DNA was blotted onto a Hybond N^+ membrane (Amersham) that was used according to the manufacturer's recommendations. Hybridization of Hybond N^+ was performed in flasks in a Hybaid oven at 65°C. DNA probes, labeled with [α-^{32}P]dCTP (specific activity: 0.7–2.0 × 10^9 dpm/μg), were obtained using the mixed primer method (Boehringer Mannheim).

RESULTS

Homologous Recombination Between Target Locus and Repair Construct pSDM321. Cocultivation of 3.6 × 10^7 protoplasts of plant line T, which is hemizygous for the target locus depicted in Fig. 1, with *Agrobacterium* strain SDM321 resulted in 109 kanamycin-resistant calli. This is a number 6000- to 7000-fold lower than that obtained in parallel cocultivation experiments with control strain SDM320 (contains a T-DNA with an intact Km^r gene, Fig. 1), which provided an indication for the total number of transformed survivors in the targeting experiments, ≈7.0 × 10^5. The 109 kanamycin-resistant calli were screened by PCR analysis with primer set 1 and 10 for the occurrence of homologous recombination. With this primer combination a 1301-bp fragment is amplified when an intact recombinant Km^r gene is present, whereas the presence of a putative contaminating control construct will result in amplification of a 1438-bp fragment (Fig. 1). In 1 of the 109 kanamycin-resistant calli transformed with construct pSDM321 a recombination product was detected. Regeneration of shoots from this recombinant callus resulted in plant line A.

The Corrected Km^r Gene in Line A Is Not Present at the Target Locus. For Southern analysis genomic DNA was first digested with *Eco*RI and *Bcl* I and then hybridized with the NPTII probe (Fig. 2). The target locus in plant line T showed a NPTII hybridizing fragment of 0.96 kbp (Fig. 3). An additional 2.0-kbp NPTII hybridizing *Eco*RI/*Bcl* I fragment, indicative of the presence of the recombinant Km^r gene, was detected in plant line A and confirmed our conclusions from the PCR analysis (see above). The presence of a 1.7-kbp *Eco*RI fragment indicated the insertion of one or more unaltered repair T-DNAs in this line (Figs. 2 and 3).

Subsequent digestion with *Hin*dIII and hybridization with the NPTII probe revealed that the 2.5-kbp fragment of the target locus was left unchanged in plant line A (Fig. 3). In case of a targeting event (T.E. in Fig. 2) this fragment should have been converted into a 3.6-kbp fragment. Instead, two extra NPTII hybridizing *Hin*dIII fragments were observed, indicating the insertion of extra T-DNAs at chromosomal positions other than the target locus. One of the *Hin*dIII fragments in line A was 2.1 kbp in size, which is indicative of the presence of an intact copy of repair construct pSDM321 (Fig. 2). The substoichiometry of the extra bands can be explained by the fact that the 2.5-kbp *Hin*dIII fragment from the target locus carries two NPTII hybridizing regions, whereas the extra fragments contain only one copy of this region. Hybridization of *Hin*dIII-digested DNA with the HPT probe indicated that also the junctions between the artificial target locus and plant chromosomal DNA, in Figs. 2 and 3 referred to as *J1* and *J2*, had been left unchanged and confirmed that two extra T-DNA copies had been inserted into the genome of line A (Fig. 3).

The exact number of integration sites (including the target locus) was determined by digestion with *Xho* I (for which no cleavage sites are present in the T-DNAs used) and hybridization with the NPTII probe. The target line (T) and the targeted line (R) isolated in our previous experiments (6) showed one NPTII hybridizing fragment of ≈20 kbp repre-

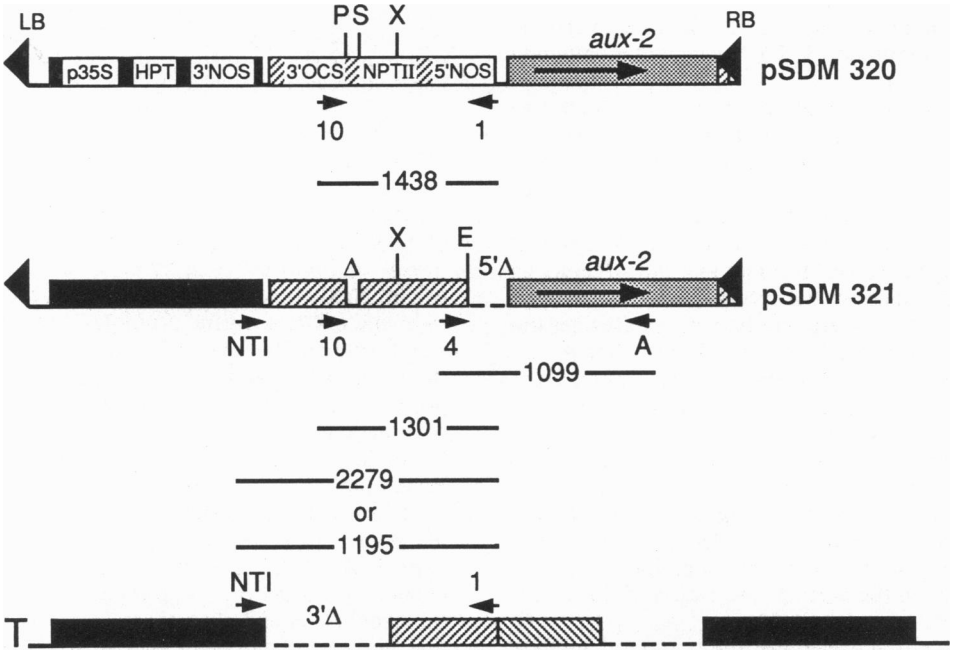

FIG. 1. T-DNA constructs pSDM320 and pSDM321, the artificial target locus in plant line T, and the primer combinations used for PCR analysis. The sizes of the fragments expected to be amplified are indicated in bp. The arrow in *aux-2* indicates the direction of transcription. p35S, promoter region of the cauliflower mosaic virus 35S transcript; HPT or NPTII, region encoding hygromycin or neomycin phosphotransferase, respectively; 5'NOS or 3'NOS, promoter or transcription termination area of the nopaline synthase gene; 3'OCS, transcription termination area of the octopine synthase gene; RB and LB, right and left T-DNA border repeat; X, *Xho* II; E, *Eco*RI; S, *Sma* I; P, *Pst* I.

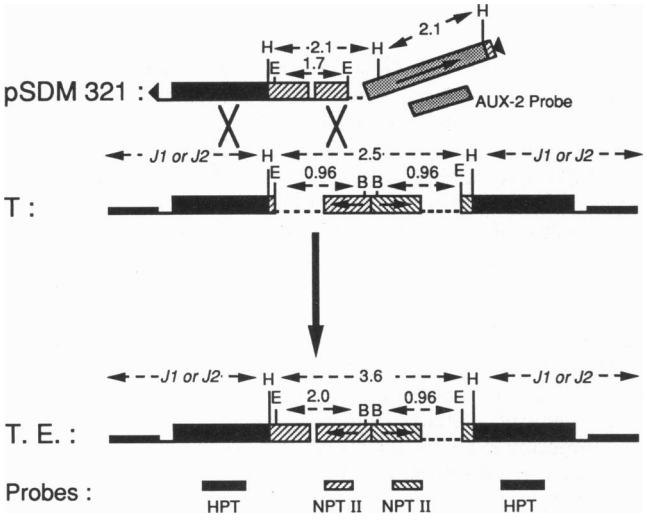

FIG. 2. Schematic representation of a gene targeting event (T.E.) after transformation of protoplasts of plant line T with construct pSDM321 (see also Fig. 1 and legend). The predicted sizes of restriction fragments are indicated in kbp and the direction of transcription of the *aux-2* gene and the 3′ deleted or corrected Km^r gene is indicated by an arrow. The HPT hybridizing junction fragments between the artificial target locus and plant genomic DNA (thick lines) are referred to as *J1* and *J2*. E, *Eco*RI; B, *Bcl* I; H, *Hin*dIII.

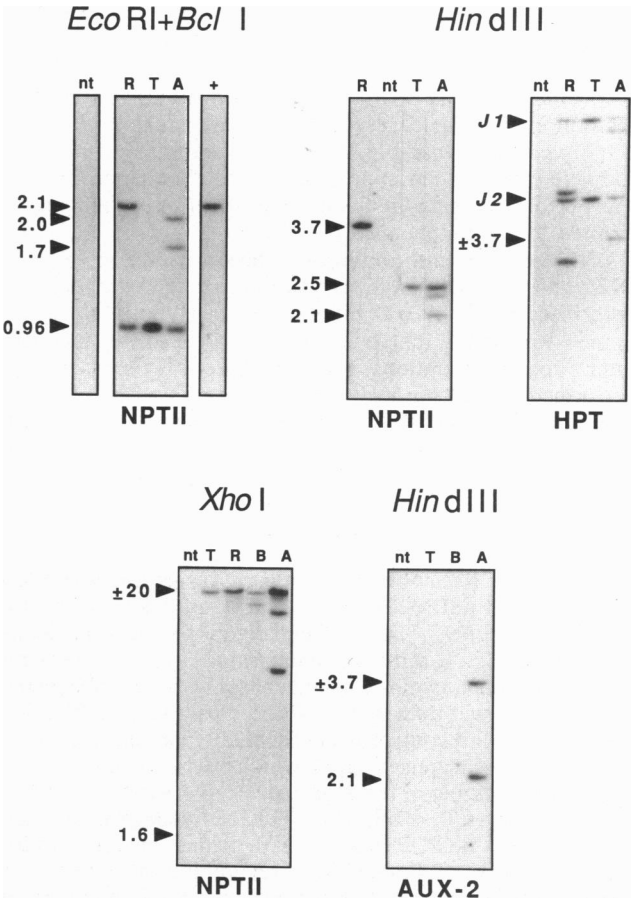

FIG. 3. Southern analysis of plant line A. Restriction endonucleases used for digestion and probes used for hybridization are indicated above and below the blots, respectively. Sizes of expected fragments (see Fig. 2) are indicated in kbp. The ± indicates that the size of a fragment was estimated using a DNA size marker as reference. nt, Wild-type tobacco; T, original target plant line; R, targeted plant line from previous experiments (6) was used as reference for a targeting event (T.E. in Fig. 2); +, plant line transformed with control construct pSDM300; A, recombinant plant line A; B, recombinant plant line that will be reported elsewhere.

senting the original target locus and the targeted locus, respectively. Both bands run at approximately the same location as a difference of 1.1 kbp in fragments of this large size is not resolved in the gel system used. In plant line A additional hybridizing fragments were detected (Fig. 3), which indicated that the two extra T-DNAs had been inserted at separate locations either on the same chromosome or on different chromosomes. Finally, a blot containing *Hin*dIII-digested DNA of line A was hybridized with the AUX-2 probe. The 2.1-kbp fragment confirmed the presence of an unaltered copy of the repair T-DNA (Figs. 2 and 3) and an extra AUX-2 hybridizing fragment of ≈3.7 kbp suggested the integration of one additional T-DNA segment containing *aux-2* sequences (Fig. 3).

Thus, homologous recombination between the incoming T-DNA and the target locus did occur in line A, but the target locus was left unchanged. Apparently, it was not the defective Km^r gene at the target locus that was corrected after recombination but rather the defective gene of the incoming repair construct. The corrected construct was inserted at another chromosomal location. The fact that the target locus remained unaltered indicated that recombination occurred via a nonreciprocal gene conversion-like process. Besides the target locus and the corrected Km^r T-DNA copy, line A was found to contain one unchanged copy of the repair construct pSDM321 at a different chromosomal location and an extra insert containing *aux-2* sequences.

Sequence Analysis of the Recombination Product. The 1301-bp recombination product obtained from plant line A by PCR amplification with primer combination 1 and 10 was cloned and sequenced. No deletions, insertions, or base-pair mutations were detected, indicating that homologous recombination had resulted in the perfect restoration of the defective Km^r gene on the incoming DNA.

To analyze the upstream region of the corrected T-DNA we performed inverse PCR as depicted in Fig. 4*A*. Inverse PCR on DNA from plant line A showed amplification of one fragment of ≈1 kbp (IPCRA), whereas no amplification products were found with DNA from line T or line R (not shown). Fragment IPCRA was cloned and sequenced (Fig. 4*B*). The entire 5′ region comprising the nopaline synthase (*nos*) gene promoter is present upstream of the restored T-DNA copy, lacking only the first 4 bp from the nick site of the right border repeat. This sequence continues with a short (5 bp) inverted repetition of the end of the *nos* gene promoter (arrow 3) followed by a 15-bp DNA segment of unknown origin. Next comes a perfect 43-bp inverted repetition of the right end of the *nos* gene promoter up to the *Bcl* I restriction site (arrow 1). The inverted repeat structure is reminiscent of the inverted repeat structure at the original target locus. Its presence suggests that in addition to the 5′ end of the resistance gene part of the inverted repeat structure of the target locus was copied to the incoming repair T-DNA. Remarkably, the 43-bp repetition is followed by a short stretch of multiple cloning sites and the 3′ noncoding region of the *aux-2* gene from the *Hin*dIII site to the *Hpa* II site [base pairs 3390–4076, according to Barker *et al.* (9)]. This sequence (including the 43-bp repetition) is identical to that of the right border end of repair construct pSDM321, suggesting that this part of the incoming construct including the *aux-2* gene was inverted during conversion of the repair construct by the target locus.

These sequence data are completely in accordance with the Southern blot data. The size of the NPTII hybridizing *Hin*dIII fragment from the corrected construct, which was estimated

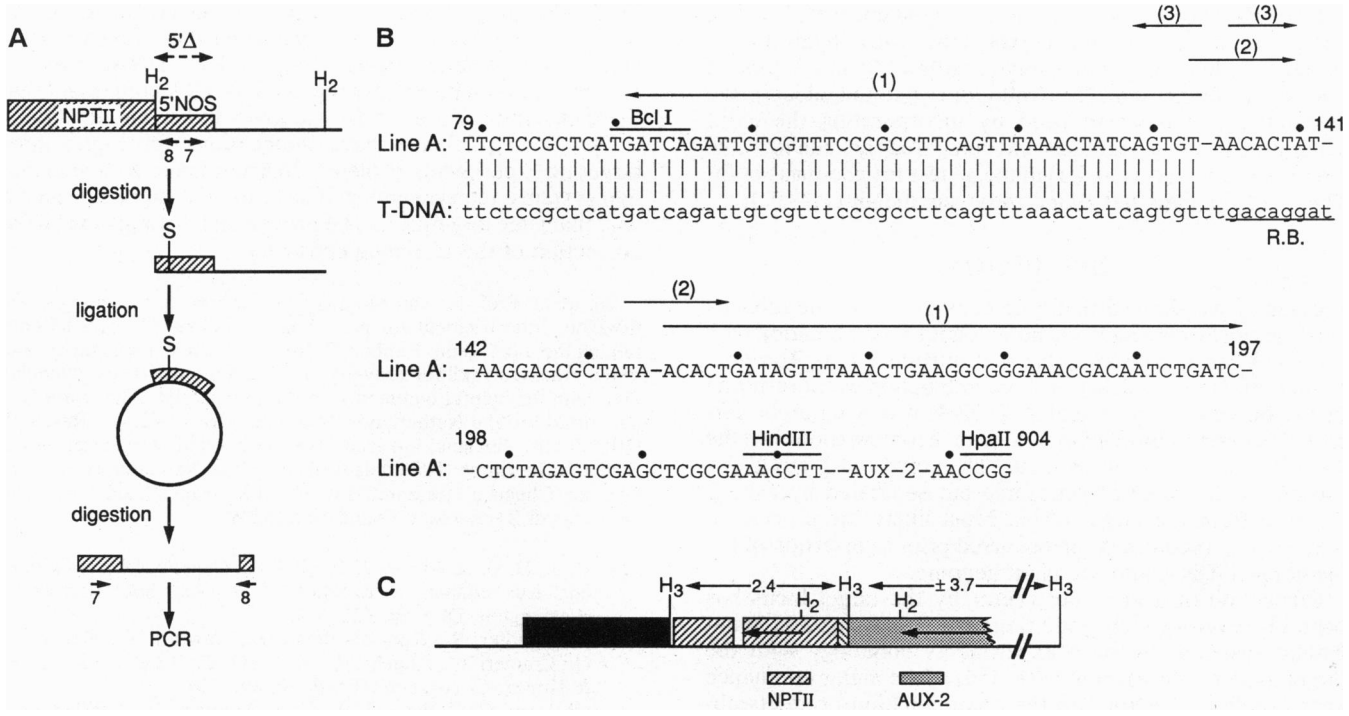

FIG. 4. Sequence analysis of the upstream region of the corrected T-DNA insert in line A. (*A*) Depiction of the inverse PCR method. Primers 7 and 8 are positioned within the 5′ region deleted from the repair construct pSDM321. The position of this region is indicated. H_2, *Hpa* II; S, *Sac* II. (*B*) Sequence of the inverse PCR fragment from plant line A is aligned with the sequence of the right border end of T-DNA construct pSDM104 (6) that is present at the target locus (T-DNA). The first eight bases of the 25-bp right border repeat (R.B.) are underlined. Base numbering 79–904 is from the first base of primer 7. The arrows numbered 1–3 indicate the positions of repeats in the sequence. AUX-2 between the *Hin*dIII and the *Hpa* II sites indicates the presence of the 3′ noncoding region of the *aux-2* gene from position 3390 to position 4076 (9). (*C*) Structure of the corrected incoming repair T-DNA as deduced from the Southern blot and sequence data. Sizes of the *Hin*dIII (H_3) fragments hybridizing to the NPTII or AUX-2 probe are indicated in kbp. H_2, *Hpa* II.

to be 2.4 kbp from the Southern analysis, was now calculated to be exactly 2418 bp (Fig. 4*C*). Moreover, the finding that *aux-2* sequences are present upstream of the corrected construct does explain the detection of one additional band hybridizing to the AUX-2 probe in the Southern analysis. The fact that the length of the hybridizing fragment is ≈3.7 kbp and not 2.1 kbp (Fig. 2) indicates that the 5′ part of the *aux-2* fragment is deleted.

Linkage Analysis of the T-DNA Insertions in Line A. Seeds obtained after selfing of plant line A were germinated on medium containing kanamycin or hygromycin. Resistance to kanamycin segregated at a 3:1 ratio (Table 1), as was expected from the presence of one recombinant Km^r gene. Hygromycin resistance (Hm^r) showed a segregation ratio that suggested the presence of two unlinked loci. Molecular analysis of line A, however, clearly predicted three separate T-DNA insertion loci containing a Hm^r gene: the target locus, the corrected incoming construct, and one additional repair construct.

In a more detailed study of the linkage between these insertions we performed PCR analysis on DNAs of 21 kanamycin-resistant seedlings using different primer combinations, each specific for a T-DNA insertion locus (Fig. 1). With primer combination 1 and 10 the corrected Km^r gene (1301-bp fragment) was detected in each of the 21 seedlings (Table 2). The 1195-bp fragment that is specifically amplified with primer combination 1 and NTI from the target locus was obtained in 18 of the 21 Km^r seedlings (genotypes I and II, Table 2). The observed ratio of 6 PCR positive to 1 PCR negative seedlings (Table 2) suggests that the assortment of the target locus and the corrected T-DNA is not completely independent, which is in accordance with the segregation data of Hm^r. Thus, the target locus and the corrected T-DNA seem to be present on the same chromosome. However, in this case the two loci must be separated by a large distance as segregation is detectable in 3 of the 21 Km^r seedlings.

PCR analysis with primer combination A and 4 showed amplification of a 1099-bp fragment in 15 of the 21 tested seedlings, indicating the presence of an unchanged repair construct in these plants (genotypes I and IV, Table 2). This insert and the recombinant locus showed an independent assortment typical for unlinked loci ($3^+:1^-$; significant in the binomial test at the 5% level). The target locus and the extra insert of the repair T-DNA also showed an independent assortment (14 of genotype I:4 of genotype II, Table 2), suggesting that there is no linkage between these loci either.

Table 1. Analysis of progeny from a selfing of line A

Selection	Res./sens.*	Ratio†	χ^2
Kanamycin	242:65	3:1	2.39
Hygromycin	286:17	15:1	0.21

*Ratio of resistant to sensitive seedlings.
†The data obtained were tested for goodness of fit with the presented ratio in the χ^2 test and were found to be significant ($P > 0.1$ and $P > 0.6$, respectively).

Table 2. PCR analysis of the genotype of 21 kanamycin-resistant seedlings obtained after selfing of line A

Primers	Locus	Genotype I	II	III	IV	Ratio +:−
1 + 10	Recombinant	+	+	+	+	21:0
1 + NT	Target	+	+	−	−	6:1
A + 4	Repair (321)	+	−	−	+	2.5:1
No. of seedlings per genotype		14	4	2	1	

The primer sets used are depicted in Fig. 1.

Since the target locus and the corrected construct are not completely linked, one would expect the ratio of hygromycin-resistant to hygromycin-sensitive seedlings from a selfing of line A to be higher than 15:1 (ratio for two unlinked loci). We calculated the expected ratio by incorporating the extra separation in 1 of 7 seedlings and compared the data in Table 1 with this new ratio (20.8:1) in the χ^2 test for goodness of fit. The fit of the data was found to be significant ($P > 0.3$).

DISCUSSION

Previously we showed that a defective locus in the tobacco genome can be corrected via homologous recombination with a T-DNA repair construct that was introduced via *Agrobacterium* (6). Here we describe a recombination event resulting in the opposite. An incoming T-DNA was accurately corrected via nonreciprocal homologous recombination with the T-DNA insert at the target locus. The corrected T-DNA was present at the same chromosome but separated by a large distance from the target locus. Most likely the process of homologous recombination occurred prior to insertion of the incoming T-DNA into the plant genome.

Correction of a targeting vector by the target locus has been observed in early gene targeting experiments in mammalian systems. In these experiments homology with the target locus was present at both sides of the mutant sequence in the targeting vector and the recombination products obtained fit the predictions of the double-strand break repair (DSBR) model for recombination (15, 16). In contrast, the recombination event presented in this paper is not easily explained by the DSBR model since the homology between the target locus and the region right to the mutant sequence in construct pSDM321 comprises only 50 bp. Moreover, this small region of homology is separated from the 600-bp homologous region to the left of the mutation by an ≈2.3-kbp nonhomologous sequence (the *aux-2* gene, Fig. 1). Data obtained more recently in mammalian systems suggest that sometimes sequences beyond the region of homology are copied from the target locus to the incoming construct (17, 18). An alternative mechanism has been proposed to explain this recombination process. The mechanism resembles DSBR in that the recombination is initiated by invasion of one of the 3′ OH strands of the acceptor DNA molecule into the homologous donor duplex. Subsequently, the invading strand is elongated using the complementary chromosomal strand as template (17, 18). In view of the structure of the corrected T-DNA insert and the homology distribution between repair construct pSDM321 and the target locus, a 3′ OH strand invasion and elongation model could provide a plausible explanation for the initiation of repair of the pSDM321 T region by the target locus. However, the T-DNA transfer intermediate is assumed to be a single-stranded linear DNA molecule with its 3′ OH end at the left border site (19), and thus 3′ OH elongation toward the right border site can occur only during or after synthesis of the second T-DNA strand. The inversion of the right end of the repair T-DNA containing *aux-2* sequences could then be due to the interaction of the 50-bp sequence at the right end of the T strand with the inversely orientated homologous sequence at the target locus during the recombination process.

According to this model the inverted repetition upstream of the corrected T-DNA represents the junction between the invertedly inserted T-DNAs at the target locus in plant line T. Multiple T-DNA inserts present at one locus are often found to be organized as head-to-head (inverted) concatemers as in target line T (20–22). The DNA sequences between such invertedly linked T-DNAs have not yet been determined and thus we do not know by which processes these repeats arise. Our sequence data suggest that "filler" DNAs may be present between invertedly repeated T-DNA copies and that these structures do not arise via plain end-to-end ligation.

Our findings clearly have implications for future gene targeting experiments in plants. In accordance with mammalian systems, experiments will have to be designed in such a way that gene targeting can be proven and distinguished from correction of the incoming construct.

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