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Integration of an Insertion-Type Transferred DNA Vector from *Agrobacterium tumefaciens* into the *Saccharomyces cerevisiae* Genome by Gap Repair

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Recently, it was shown that *Agrobacterium tumefaciens* can transfer transferred DNA (T-DNA) to *Saccharomyces cerevisiae* and that this T-DNA, when used as a replacement vector, is integrated via homologous recombination into the yeast genome. To test whether T-DNA can be a suitable substrate for integration via the gap repair mechanism as well, a model system developed for detection of homologous recombination events in plants was transferred to *S. cerevisiae*. Analysis of the yeast transformants revealed that an insertion type T-DNA vector can indeed be integrated via gap repair. Interestingly, the transformation frequency and the type of recombination events turned out to depend strongly on the orientation of the insert between the borders in such an insertion type T-DNA vector.

Agrobacterium tumefaciens is a soil bacterium which causes crown gall disease in a wide range of dicotyledonous plants. Tumor growth is induced by the transfer and integration into the plant genome of a segment of transferred DNA (T-DNA), which is part of the Ti plasmid in the bacterium. The T-DNA contains genes encoding enzymes for phytohormone production and opine synthesis. It is transferred as a single-stranded DNA molecule (T strand). The transfer process is determined by the virulence (*vir*) genes that are located on the Ti plasmid and are induced by phenolic compounds such as acetosyringone (21, 46). The VirD2 protein nicks the Ti plasmid at the T-DNA border repeats with help of VirD1 (38) and becomes covalently attached via its tyrosine 29 to the thymidine at the very 5′ terminus of the T strand (43). Subsequently, the T strand, with VirD2 attached to it, is released and transmitted to the plant cell (41, 42). The VirD2 protein has a nuclear localization sequence in the C-terminal part, which directs the T strand to the nucleus (9, 17, 35, 41, 44, 45). The single-stranded-DNA-binding protein VirE2 can coat the entire T strand in vitro (5) and may protect it against degradation by nucleases (36). The VirB proteins probably form a structure allowing the transfer of the T strand with the attached proteins (T complex) to plant cells (3).

The *Agrobacterium* DNA transfer system has been used extensively for the incorporation of new genetic traits into the plant genome. The T-DNA integrates predominantly via illegitimate recombination (12, 25, 26, 46) but can integrate via homologous recombination as well, although at low frequencies (23, 28, 30, 32). Recently, it was shown in our laboratory that *A. tumefaciens* can transfer T-DNA not only to plants but also to the yeast *Saccharomyces cerevisiae* (4). Molecular analysis of the T-DNAs that had integrated into the yeast genome revealed that they had integrated via homologous recombination.

Two types of DNA vectors are used for gene targeting purposes, namely, replacement and insertion vectors. These types of targeting vectors are thought to integrate via different mechanisms. A replacement vector is likely to integrate nonconservatively, i.e., via double crossover or via gene conversion replacing part of the target locus. Integration of an insertion vector seems to occur according to the double-strand-break repair model and is conservative (40). This model predicts 5′→3′ exonucleolytic activity at the homologous ends of the insertion vector followed by invasion and annealing of the single-stranded ends to the homologous target. The gap which arises from the exonuclease activity is repaired via gene conversion on the homologous template, resulting in two Holliday structures (19). Resolution of the single crossover can lead to recombination of the two DNA molecules and integration of the entire vector. Gene-targeting experiments in mammalian cells have shown that an insertion type vector recombines with a higher efficiency than a replacement type vector depending on the topology of the vector (16) and the position of the target locus (15).

A T-DNA can be used as a replacement type vector, but so far no positive results have been obtained when it has been used as an insertion type vector in plants. In this study, we investigated whether a T-DNA can be used as an insertion type vector to be integrated via homologous recombination according to the double-strand-break repair model. It can be envisaged that the *vir* proteins and especially VirD2 attached to the right border end may obstruct the removal of the nonhomologous borders and thus the repair of the gap via a gene conversion reaction. Our finding that T-DNA integrates efficiently into the yeast genome via homologous recombination was exploited for the analysis of the recombination products. This study shows that a T-DNA can be integrated via gap repair and that the nonhomologous borders and the VirD2 protein do not obstruct the gap repair reaction. Unexpectedly, the transformation frequency and the type of recombination turned out to depend strongly on the location of the right T-DNA border.

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MATERIALS AND METHODS

Plasmid constructs. (i) Target construct. The target construct (see Fig. 1) consisted of four segments: a defective *uidA-nptII* fusion gene and a *hpt* gene

cloned between two right T-DNA (T_R-DNA) fragments which originated from Ti plasmid pTi15955 (1). The T_R-DNA sequences were subcloned from pRAL3267 containing the *Bam*HI-2 fragment from pTi15955. The small T_R-DNA fragment was a 1,262-bp *Pst*I-*Rsa*I fragment containing the agropine synthase (2′) gene and was cloned in pIC20R, resulting in a *Bgl*II-*Bam*HI fragment. The large T_R-DNA fragment was a 3,984-bp *Sac*I-*Pst*I fragment and contained the mannopine synthase (0′ and 1′) genes. This fragment was cloned in pIC19R, resulting in a *Kpn*I-*Bam*HI fragment. This *Bam*HI site was used for insertion of the other three segments, eventually resulting in construct pSDM4037.

The *hpt* gene was constructed from pTRA151 (47) and pNOS-HPT (13). The 35S promoter from pTRA151 was replaced by the *nos* promoter from pNOS-HPT. The two *Bam*HI sites flanking the *hpt* open reading frame were removed by filling in. Subsequently, *Bgl*II and *Bam*HI sites outside the gene were obtained by subcloning with pBluescript KS^+ in pIC20R. During further cloning in *Escherichia coli*, the *hpt* gene could be used as an additional selectable marker allowing selection on hygromycin at 100 mg/liter.

The *uidA-nptII* gene was obtained from pBI426 (6). The internal *Bam*HI and *Bgl*II sites were removed by filling in to facilitate further cloning. New *Bgl*II and *Bam*HI sites outside the gene were obtained by subcloning with pBluescript KS^+ in pIC20R. The 35S promoter and 90 bp of the *uidA* gene up to the *Bcl*I site were deleted by cloning the *Bcl*I-*Bam*HI fragment.

After the three fragments were cloned into the *Bam*HI site behind the large T_R fragment, the entire *Kpn*I-*Bam*HI insert from pSDM4037 was cloned between the *Kpn*I-*Bam*HI sites of the yeast shuttle vector pUT332 (11), leading to a loss of the *TEF1* promoter and the coding region of the Tn*5*-phleomycin resistance gene. The resulting replicative vector, pSDM4200, contained the *URA3* marker gene and 2 μm origin of replication for selection and replication in *S. cerevisiae*. For stable integration of the target construct into the yeast genome, the insert from pSDM4037 was cloned into two *Bam*HI sites located next to the *URA3* gene of the integrative vector pUC4-α10::*ura3* (39). A *Bam*HI linker was inserted at the *Kpn*I site of pSDM4037 to facilitate cloning. In the resulting integrative vector pSDM4208, the target construct and the *URA3* gene were flanked by 1.8 and 1.5 kb, respectively, of yeast genomic DNA from the *PDA1* locus on chromosome V.

(ii) Targeting vectors. The insertion type targeting vectors consisted of six *Bgl*II-*Bam*HI segments: the two T_R-DNA fragments, the *hpt* gene, a 3′ subfragment of the *uidA-nptII* fusion, a 5′ subfragment which was linked to the *LEU2* gene, and a streptomycin resistance gene (see Fig. 1). The *Bcl*I and *Bgl*II sites were removed from the large and small T_R fragments, respectively, for better discrimination between the target T-DNAs and the targeting vectors (see Fig. 4B). The *hpt* gene was identical to that of the target T-DNAs.

The streptomycin resistance (*spt*) gene was obtained from pSLJ1491 (a kind gift of J. Jones, The Sainsbury Laboratory, Norwich, United Kingdom). The 35S promoter was exchanged for a *Bcl*I-*Bgl*II fragment of pFWP101 (37) containing the figwort mosaic virus 34S promoter. The *spt* gene with the 34S promoter, an *ocs* terminator, and about 600 bp of Tn*5* sequences were subcloned in pIC20H and then cloned as a *Bcl*I-*Bam*HI fragment in the targeting vector.

The *uidA-nptII* gene was split at an *Asu*II site in the *uidA* open reading frame. The 3′ *Asu*II-*Bam*HI part was subcloned in pIC20H. A frameshift mutation was introduced in the *nptII* coding region by filling in the *Nco*I site, resulting in a new *Nsi*I site (see Fig. 2 and 4). The 5′ part ended 25 bp upstream of the *Asu*II site at which a gap was introduced and was cloned by PCR amplification of a 483-bp fragment up to the gap with primers gus (5′-GTCGCTCGAGACTGTAACCA CGCGTCTG-3′) and gus2 (5′-GGTTGGATCCGACAGCAGTTTCATCAAT C-3′). This fragment was fused to the remaining 5′ part of the *uidA-nptII* gene. Because the *uidA-nptII* gene was not suitable for G418 selection in *S. cerevisiae*, the 35S promoter up to the *Bcl*I site in the *uidA* open reading frame was exchanged for a 2.7-kb *Sal*I fragment from pYY27 containing the *LEU2* gene (18). The six *Bgl*II-*Bam*HI segments were linked head to tail, and the final *Bgl*II-*Bam*HI insert of pSDM4066 was cloned in the binary vector pSDM14 (29) in both orientations between the T-DNA borders, resulting in pSDM4204 and pSDM4205. In pSDM4204, the right T-DNA border was linked to the right arm of the insertion type vector, whereas in pSDM4205, the right border was on the left arm adjacent to the *LEU2* gene.

***Agrobacterium* strains.** The binary vectors pSDM4204 and pSDM4205 were electroporated to *A. tumefaciens* LBA1100 (2), which contains the helper plasmid pAL1100 lacking the T region and the *tra* region. Deletion of the transfer (*tra*) genes made DNA transfer dependent on the virulence (*vir*) genes located on the helper plasmid. *A. tumefaciens* SDM4204 and SDM4205 were used as T-DNA donors for the yeast target strains Y4200 and Y4208.

Yeast strains. The vectors pSDM4200 and pSDM4208 were electroporated to the haploid yeast strain M5-1a (*MAT***a** *trp1-92 leu2-3/112 ura3-52 his4*). Transformants were selected for the presence of the *URA3* gene on minimal MY medium (48) complemented with histidine, tryptophan, and leucine. The plasmid present in the resulting strain Y4200 was checked after isolation and transformation to *E. coli*. Proper integration of pSDM4208 into the yeast genome was confirmed by Southern blot analysis. Strain Y4208, in which the entire circular pSDM4208 vector had integrated at the 1.8-kb homologous segment of the *PDA1* locus via a single crossover, was selected.

T-DNA transfer to *S. cerevisiae*. The selected yeast target strains Y4200 and Y4208 were used as recipients of T-DNA from *A. tumefaciens* SDM4204 and SDM4205 containing the binary vector pSDM4204 or pSDM4205, respectively. Coincubation was done on filters for three days as was described previously (4). To select for maintenance of the replicating plasmid in Y4200 and to compare recombination in Y4200 with that in Y4208, the target yeast strains in experiments I, III, and IV were grown on minimal medium.

PCR analysis. The PCRs were performed in a Perkin-Elmer model 480 thermocycler. Genomic DNA (1 μg) was used in the PCR with 0.1 U of *SuperTaq* polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom) in a total volume of 100 μl, using a program of 30 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min. An elongation period of 5 min instead of was used for PCR fragments larger than 1.5 kb. Yeast plasmid preparations were made by the method of Robzyk and Kassir (33), and genomic DNA was isolated with lyticase. Cells were collected from a 2-ml culture, 80 μl was added from a solution containing 1 M sorbitol, 0.1 M EDTA, 0.15 M NaCl, and 4 mg of lyticase per ml, and the mixture was incubated at 30°C for 30 min. After centrifugation, the pellet was resuspended in 240 μl of 10 mM Tris–20 mM EDTA−1% sodium dodecyl sulfate (SDS) and incubated at 65°C for 5 min. This was followed by phenol-chloroform extractions until no interface was visible, DNA precipitation, and a 70% ethanol washing step. The dried pellet was dissolved in 50 μl of water, and 5 μl was used for PCR. The primers were gus (5′-GTCGCTCGAGACTGTAA CCACGCGTCTG-3′), gap1 (5′-GCCTAAAGAGAGGTTAAAGCC-3′), gap2 (5′-TTAACCTCTCTTTAGGCATTGG-3′), hyg (5′-GGCCTCCGCGACCGG CTGCAGAACAGC-3′), leu (5′-TCAGAAACGGCCTTACGACG-3′), bin (5′-TCTAGCCGACTTGTCCGGTG-3′), and npt (5′-ACCGTAAAGCACGAGG AAGC-3′).

Southern blot analysis. Yeast genomic DNA was isolated by the method of Holm et al. (20); 10 μg of DNA was digested with different enzymes and separated on a 0.7% agarose–89 mM Tris-borate–2 mM EDTA gel. DNA was transferred to Hybond N+ membranes (Amersham) by capillary blotting with 0.4 M NaOH. Membranes were (pre)hybridized as described in the Hybond N+ protocol. DNA probes labelled with [α-^{32}P]dCTP (specific activity, 0.5×10^9 to 1×10^9 dpm/μg of DNA) were obtained by the method described by Feinberg and Vogelstein (10). Final washing was performed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. The blots were exposed to Phosphorimager screens.

RESULTS

Time-consuming selection procedures and frequent silencing of genes hamper the study of gene targeting in plants. Nevertheless, it is very important that targeting vectors be developed for plants. It would be ideal if these could be incorporated into the highly efficient and natural gene delivery system of *A. tumefaciens*. Extrachromosomal recombination and homologous integration into the plant genome of the T-DNA have shown that the T complex, which is a nucleoprotein complex, is a suitable substrate for recombination. So far, only replacement type vectors have been used for gene-targeting experiments in plants. An insertion type vector would be an interesting alternative that might result in higher targeting frequencies in plants. In this study, the effects of the incorporation of an insertion type vector within the T-DNA was investigated with *S. cerevisiae* as a recipient of the T-DNA.

Construction of the yeast target strains. A construct being used as an artificial target locus for gene targeting in plants was introduced into the haploid yeast strain M5-1a on either a replicating vector (pSDM4200) or an integrative vector (pSDM4208) (Fig. 1A). The target construct contained a 5.2-kb segment of T_R-DNA from the Ti plasmid of *A. tumefaciens* into which a hygromycin phosphotransferase (*hpt*) gene and a promoterless *uidA-nptII* fusion gene encoding a β-glucuronidase neomycin phosphotransferase fusion protein had been cloned (see Materials and Methods). In both vectors, the *URA3* gene was used for selection of the primary transformants, resulting in yeast strains Y4200 and Y4208. The presence of the replicating vector in Y4200 was checked by plasmid rescue to *E. coli*; the presence of the integrated target construct at the *PDA1* locus in Y4208 was verified by Southern blot analysis. One strain in which the entire pSDM4208 plasmid had integrated via a single crossover at the *PDA1* locus was selected.

Construction of the insertion type T-DNA vectors. An insertion type T-DNA vector which had the selectable *LEU2* gene in front of the *uidA-nptII* gene was constructed (Fig. 1B). A

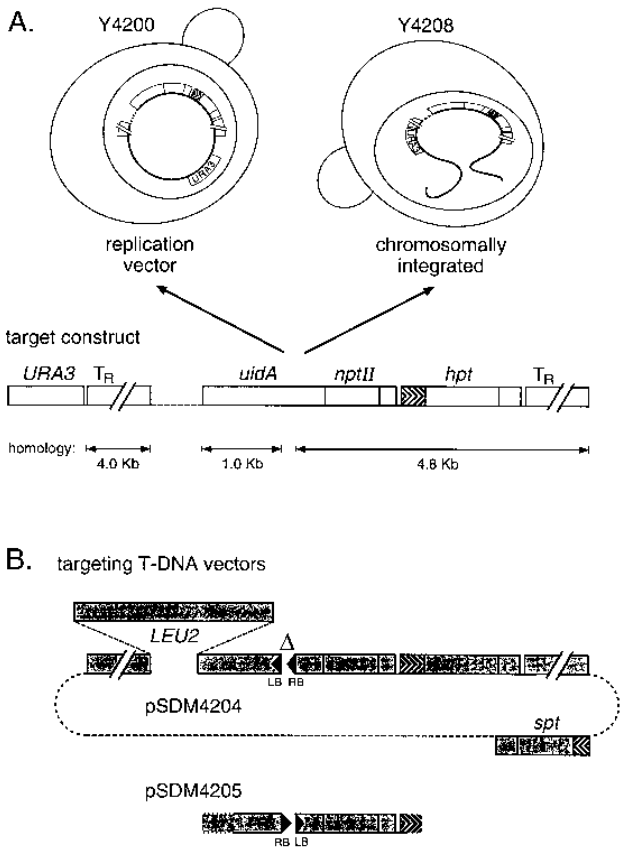


FIG. 1. Experimental design for *A. tumefaciens*-mediated transformation of *S. cerevisiae*. (A) Transgenic *S. cerevisiae* strains were selected for the *URA3* marker after electroporation of a vector containing the target construct. The target construct was located either on a replicating vector (pSDM4200) or on an integration vector (pSDM4208) which was inserted at the *PDA1* locus of the yeast genome. (B) Aligned insertion T-DNAs from *A. tumefaciens* SDM4204 and SDM4205. In pSDM4204, the right T-DNA border was linked to the right arm, whereas in pSDM4205, the right border was at the left arm, adjacent to the *LEU2* marker. Transformed yeast cells were selected for the presence of the *URA3* and *LEU2* markers. The plant gene constructs present in the T region functioned only as the areas of DNA homology. Joined DNA fragments are indicated by dotted lines. *uidA*, β-glucuronidase coding region; *hpt*, *nptII*, and *spt*, hygromycin, neomycin, and streptomycin phosphotransferase-encoding regions, respectively; ⋙, plant promoter; T_R, right T-DNA sequence from pTi15955; LB and RB, T-DNA left and right border repeats; △, 25-bp sequence which was deleted in the *uidA* region.

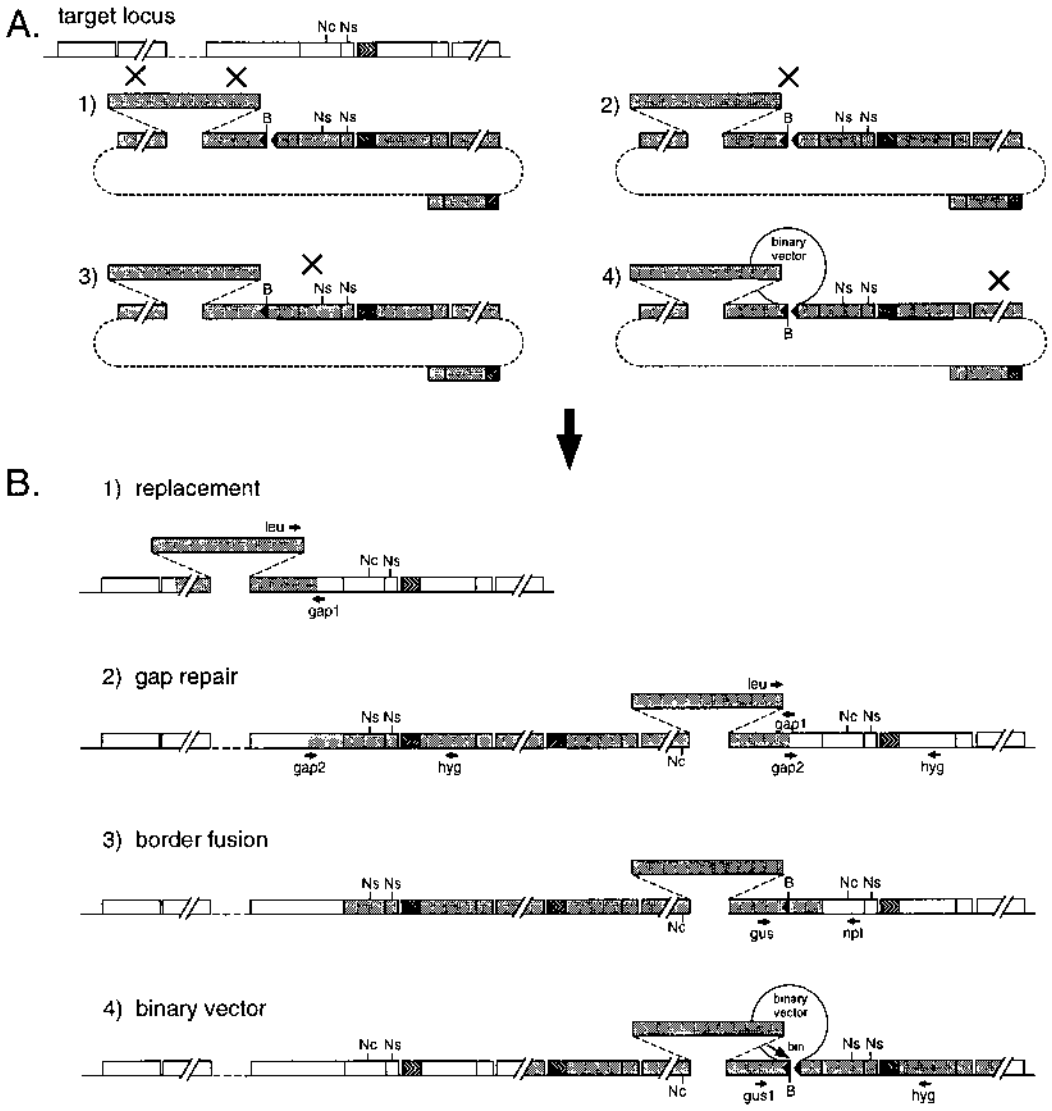
gap of 25 bp was created in the *uidA* coding region, and the ends of this gap were fused to the border repeats. The primers gap1 and gap2, which were homologous to the region lost by the gap, were used to detect gap repair events (Fig. 2B). The construct was cloned in both orientations between the left and right T-DNA borders of the binary vector, resulting in pSDM4204 and pSDM4205. The T-DNA vectors have two homologous arms: the arm to the left of the gap harbors the *LEU2* gene, whereas the right arm is entirely homologous to the target locus. In pSDM4204, the right border, to which the VirD2 protein attaches during T-DNA transfer, was located at the right arm of the insertion type vector and was 11.5 kb away from the *LEU2* gene, whereas in pSDM4205, the right border was only 1 kb away from the *LEU2* gene at the left arm of the vector. For distinction of the target DNA and the vector DNA, three restriction site substitutions were introduced into the targeting vector (see Materials and Methods and Fig. 5B). The presence of the *LEU2* gene on the left arm enabled selection of the incoming T-DNA.

T-DNA transfer from *A. tumefaciens* to *S. cerevisiae*. *A. tumefaciens* helper strain LBA1100 containing the binary vector pSDM4204 or pSDM4205 was cocultivated for 3 days with yeast strains Y4200 and Y4208 as described by Bundock et al. (4). Subsequently, the agrobacteria were killed and Leu^+ transgenic yeasts were selected by plating the mixture on selective MY medium supplemented with 100 mg of carbenicillin per liter, 200 mg of cefotaxime per liter, histidine, and tryptophan. As can be seen in Table 1, Leu^+ transfer was observed but was dependent, as expected, on induction of the *vir* genes in the donor agrobacteria by acetosyringone (Table 1). This is indicative of proper *vir*-dependent T-DNA transfer. The frequency of Leu^+ transfer tended to be higher with Y4200 than with Y4208 as a recipient. This may be because the target locus in Y4200 is present on a multicopy 2μm vector. Surprisingly, *A. tumefaciens* SDM4205 was consistently a better T-DNA donor than SDM4204. This was independent of whether the target was located on the 2μm plasmid or stably integrated in the yeast genome.

Insertion type T-DNA can be integrated via gap repair. In previous studies, it was shown that transfer of a replacement type T-DNA to *S. cerevisiae* resulted in integration of the T-DNA into the yeast genome via homologous recombination (4). In the present study, integration of the *LEU2* marker of the insertion type T-DNA could have occurred by (i) a replacement event at the left arm only, (ii) insertion of the entire T-DNA via gap repair, (iii) ligation of the left and right T-DNA borders and subsequent homologous integration of the circular T-DNA via a single crossover, or (iv) homologous integration of the entire binary vector after circularization via a single crossover (Fig. 2B). The last two events and integration via replacement were observed previously after transfer of a replacement type T-DNA to *S. cerevisiae* (4).

Leu^+ yeast strains were analyzed by PCR amplification of a fragment with primer leu, which anneals to the incoming T-DNA and gap1, which anneals to the target locus (Fig. 2A and B). In most of the tested strains, the expected 1,030-bp fragment could be amplified (results not shown), indicating that the T-DNA had integrated via homologous recombination with a crossover between these two primer sites. Other lines, however, did not show this recombinant PCR fragment and were later found to be Leu^+ as a result of integration of the circularized T-DNA (event 3) or the entire binary vector (event 4). These events do not necessarily give this fragment (Fig. 2C).

To determine in more detail how integration had occurred, the recombinant strains were screened with three more primer combinations (Fig. 2C). PCR analysis of the target yeast strains Y4200 and Y4208 with the primers gap2 and hyg resulted in a 2,543-bp fragment which could be digested by *Nsi*I into fragments of 1,785 and 758 bp. Insertion via gap repair should lead to an additional PCR fragment of the same size containing two *Nsi*I sites and resulting in fragments of 1,395, 758, and 390 bp after *Nsi*I digestion. Indeed, some of the tested yeast strains showed a PCR fragment with two *Nsi*I sites (Fig. 3). The lower intensities of the recombinant fragments which are shown for Y4200 in Fig. 3 were due to the presence of original pSDM4200 plasmids which survived selection in the yeast cells. The coexistence of the recombinant fragments with primers gap2 and hyg and with primers leu and gap1 showed that the 25-bp gap had been repaired by a conversion reaction followed by recombination and integration of the T-DNA vector (event 2). Thus, a T-DNA insertion vector is a suitable substrate for homologous integration via gap repair, and this is not prevented by the presence of the nonhomologous T-DNA border



C.

primers	predicted PCR fragments					
	replacement	gap repair	border fusion		binary vector	
			a	b	a	b
leu + gap1	+	+	+	-	+	-
gus + npt	+	+	+	+	+	+
x *Bam*HI	-	-	+	+	-	-
gap2 +hyg	+	+	+	+	+	+
x *Nsi*I	-	+	-	+	-	+
gus/hyg + bin	-	-	-	-	+	+

FIG. 2. Classification of recombinant yeast lines by PCR. (A and B) The recombinants were classified in four groups: group 1, replacement on the left arm; group 2, gap repair integration and removal of the nonhomologous T-DNA ends; group 3, insertion of a circularized T-DNA; and group 4, insertion of the entire binary vector. (B) Four primer combinations were used for classification of the recombination products. Characteristic primer combinations are indicated for each group. Primers gap1 and gap2 annealed to a 25-bp sequence which was absent in the targeting vectors. The bin primer was located outside the T region and next to the right border of the binary vector. B, *Bam*HI; Ns, *Nsi*I; Nc, *Nco*I. (C) The origin of the PCR fragments obtained with primers gus plus npt and gap2 plus hpt, respectively, was determined by the presence (+) or absence (−) of the *Bam*HI and the *Nsi*I sites which are characteristic for the targeting vector. Border fusion events and binary vector integrations result in two possible combinations of PCR fragments a and b depending on the position of the crossover.

TABLE 1. T-DNA transfer frequencies from *A. tumefaciens* to *S. cerevisiae*

S. cerevisiae strain	*A. tumefaciens* strain	AS[a]	No. of URA^+/LEU^+ colonies per output no. of yeast cells in expt:				Mean ratio[b]
			1	2	3	4	
Y4200 plasmid	SDM4204	+	$<5.7 \times 10^{-5}$ (16.3)		3.0×10^{-8} (11.1)	1.0×10^{-6} (28.3)	18.6
		−	$<5.6 \times 10^{-6}$		$<1.3 \times 10^{-8}$	$<7.4 \times 10^{-8}$	
	SDM4205	+	3.5×10^{-4} (100)		2.7×10^{-7} (100)	3.7×10^{-6} (100)	100
		−	$<5.6 \times 10^{-6}$		$<1.2 \times 10^{-8}$	$<5.3 \times 10^{-8}$	
Y4208 genome	SDM4204	+		6.0×10^{-9} (1.4)	5.5×10^{-8} (26.2)	1.5×10^{-8} (22.5)	15.7
		−		$<6.2 \times 10^{-8}$	$<1.1 \times 10^{-8}$	$<1.5 \times 10^{-8}$	
	SDM4205	+		4.2×10^{-7} (100)	2.1×10^{-7} (100)	6.5×10^{-8} (100)	100
		−		$<4.4 \times 10^{-8}$	$<8.2 \times 10^{-9}$	$<1.4 \times 10^{-8}$	

[a] AS, acetosyringone.
[b] Ratios in parentheses are relative to SDM4205.

sequences and the VirD2 protein which is bound to the right border.

Homologous integrations of substrates containing ligated T-DNA borders (event 3) were detected with primers gus and npt. After border fusion, the 2,054-bp PCR fragment overlapping the gap position has a diagnostic *Bam*HI site, whose cleavage results in fragments of 506 and 1,548 bp (Fig. 3). Recombination may have occurred via a single crossover of the circularized vector, but recombination via a double crossover of two linked T-DNAs cannot be excluded.

Primer bin, which anneals to the overdrive sequence next to the right T-DNA border of the binary vector, was used in combination with either gus or hyg (depending on the T-DNA orientation) to find evidence for the homologous integration of the entire binary vector (event 4). In this case, T-strand synthesis has skipped the left border, resulting in transfer of the whole binary vector.

DNA adjacent to the right border of the T-DNA is integrated at a higher efficiency. Integration via all four possible events was encountered by PCR (Table 2). For comparison of the two targeting vectors, the frequency of each group was corrected for the mean ratio between the transfer efficiency of SDM4204 and SDM4205 (Table 1). These ratios showed that the frequency of T-DNA transfer was sixfold higher when the *LEU2* gene was adjacent to the right T-DNA border in SDM4205. The higher transfer frequency with SDM4205 was due predominantly to extra replacement events, whereas the numbers of other types of insertions were not strikingly different for SDM4204 and SDM4205. This was the case for both yeast recipient strains. Apparently, the arm linked to the right T-DNA border of the vector was integrated at a higher efficiency. The fact that a replacement event needs only one arm of the vector for integration of the *LEU2* gene makes this event dependent on the position of the right border. The other events need both arms for recombination and thus are independent of the orientation of the T-DNA vector.

Southern blot analysis of the recombinants. For more detailed analysis of the recombination events, we focused on those that occurred in the yeast target strain Y4208, with one chromosomal copy of the target locus. Genomic DNA preparations from eight selected Leu^+ derivatives of Y4208 were digested with two pairs of restriction enzymes and hybridized with a *uidA* probe (Fig. 4). Fragments observed on the Southern blot were in agreement with the PCR data.

Recombinant strain 4205-28 showed a restriction pattern indicative of a replacement event at the left arm of the targeting vector (Fig. 4C). The preservation of the *Bcl*I site in the T_R-DNA region of the insertion type T-DNA indicated that one of the crossovers had occurred in the 3.1-kb T_R-DNA region left of the *Bcl*I site. Southern blot analysis of nine other replacement events resulted in one recombinant line with a crossover in the 0.9-kb T_R region to the right of the *Bcl*I site, indicating that most crossovers had occurred in the larger left part of the T_R-DNA (results not shown).

In recombinant clones 4204-5 and 4205-4/16/20/31, the T-DNA vector had been inserted via gap repair (Fig. 4C). The absence of the *Kpn*I site at the right border confirmed the PCR results showing that these nonhomologous sequences had been removed. The resulting gap had been repaired via gene conversion followed by resolution of the crossover and insertion of the T-DNA vector. The clones 4205-4/20/31 showed the same expected restriction pattern on Southern blots (Fig. 4A). PCR analysis showed that in clones 4204-5 and 4205-16 the *Nsi*I site in the *nptII* coding region of the targeting vector had been copied to the *nptII* sequence at the target locus, thereby removing the *Nco*I site (results not shown). The absence of the *Nco*I site was confirmed in these strains by more extensive Southern blot analysis (Fig. 4A and C). Presumably, the *Nsi*I flag had been copied via a mismatch repair reaction prior to resolution of the crossover. The restriction map of 4204-5 suggested that the gap repair integration had been followed by a second intrachromosomal recombination event in the 0.9-kb T_R-region between the *Bcl*I site and the *LEU2* gene. It is likely

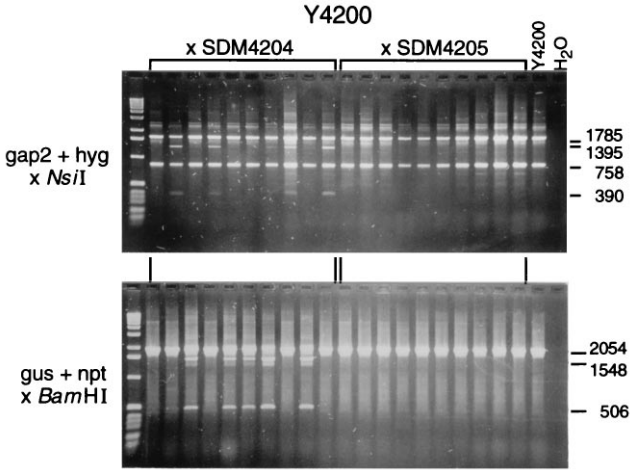


FIG. 3. Ethidium bromide-stained gels showing PCR fragments indicative for gap repair (gap2 + hyg) and border fusion events (gus + npt).

TABLE 2. Frequencies of the different recombination events

S. cerevisiae strain	*A. tumefaciens* strain	No. (frequency) of transformants[a]					
		Replacement	Gap repair insertion	Border fusion integration	Binaryvector integration	Other	Total
Y4200 (plasmid)	SDM4204	12 (7.2)	10 (6.0)	8 (4.8)	1 (0.6)		31 (18.6)
	SDM4205	42 (86)	5 (10)		1 (2.0)	1 (2.0)	49 (100)
Y4208 (genome)	SDM4204	2 (4.5)	1 (2.2)	3 (6.7)	1 (2.2)		7 (15.7)
	SDM4205	31 (89)	4 (11)				35 (100)

[a] Frequencies (in parentheses) include the mean ratio between SDM4204 and SDM4205 from Table 1. Yeast strains from experiments 1 to 3 were analyzed.

that this recombination had occurred during or immediately after insertion, since the long direct repeats appeared to be quite stable in the other clones, even in the absence of selection for the *LEU2* marker.

The presence of the *Kpn*I site in the *uidA* sequence of 4204-2 confirmed that fusion of the left to the right T-DNA border had occurred before integration. This agreed with the presence of the *Bam*HI site in the PCR fragment obtained with primers gus and npt. A circularized T-DNA had probably been integrated via a single crossover between the border fusion and the *Nsi*I site in the *nptII* gene followed by an intrachromosomal recombination to the left of the *LEU2* marker like in 4204-5.

Integration of the entire binary vector had occurred in clone 4204-1. The position of the *Bgl*II site suggested that the circularized vector had been inserted via a single crossover in the far-right 0.8-kb T_R region.

DISCUSSION

A. tumefaciens-mediated T-DNA transfer to *S. cerevisiae* has been shown to be a useful model system to study homologous recombination of the T-DNA (4). T-DNA integration in plants occurs predominantly via illegitimate recombination. The fact that T-DNA integrates via homologous recombination in *S. cerevisiae* shows that the host and not the T-DNA transfer apparatus from *A. tumefaciens* determines the nature of the integration event. Whereas the efficiency of homologous T-DNA integration is different between plants and yeasts, the analysis of mutations in the *vir* genes of *A. tumefaciens* revealed that the products of these genes are equally important for T-DNA transfer to both organisms. For instance, the deletion of the NLS sequences from the C-terminal part of the VirD2 protein, which are important for T-DNA transfer to plants, abolished transfer to *S. cerevisiae* as well. This indicates that the VirD2 protein, which is covalently attached to the T strand, directs the T-DNA to the nucleus in *S. cerevisiae* as well (4).

In this study, we tested whether the *A. tumefaciens* DNA transfer apparatus would allow the use of T-DNA as an insertion type vector for gene targeting. A model system which was based on the homologous integration of an insertion type T-DNA in plants was adapted for this purpose and tested in *S. cerevisiae*. Analysis of the recombinant yeast strains obtained with this insertion vector confirmed that integration of the T-DNA into the yeast genome had occurred by homologous recombination, irrespective of whether the target locus was located on the 2μm plasmid or in the chromosome. Moreover, PCR and Southern blot analysis of the recombinant yeast strains showed that the insertion type T-DNA vector was a suitable substrate for integration by gap repair. The VirD2 protein, attached to the 5′ right border, did not obstruct the removal of the nonhomologous T-DNA ends, and the gap was repaired via gene conversion according to the double-strand-break-repair model (40). Southern blot analysis revealed that in two gap repair events, 4204-4 and 4205-16, the *Nsi*I marker mutation in the *nptII* coding region was copied from the T-DNA vector to the target locus (Fig. 4C). During gap repair, formation of heteroduplex DNA can include mismatches which are located adjacent to the 25-bp gap. The asymmetric heteroduplex DNA can be repaired in either direction by the mismatch repair mechanism in *S. cerevisiae*, although a bias exists for repair of the broken DNA strand (27, 31, 34). Remarkably, in this study, repair of the chromosome was obtained in two of five gap repair integrations.

The high frequency of replacement events in our experiments does not necessarily suggest that a T-DNA vector integrates preferentially via a replacement in general. Vector topology, such as the size of the vector, localization and distribution of homology, and position of the selection marker, has been shown to influence the outcome of recombination experiments (8, 14, 16). Therefore, this factor was kept constant in our study. A comparison of the frequency with which integration occurred via replacement and via gap repair revealed that this ratio depended largely on the orientation of the T-DNA between the border repeats. When the right T-DNA border was adjacent to the *LEU2* gene, a sixfold-higher transformation frequency was obtained, predominantly as a result of replacement events on the left arm of the targeting vector. The other T-DNA orientation resulted in a lower frequency of transfer and a similar number of replacement events and gap repair events. The *Agrobacterium* vector system mediates T-DNA transfer to plants irrespective of the sequence content of the T-DNA, and thus both insertion T-DNA vectors are expected to be transferred with the same efficiency. The T-DNA is transferred as a single-stranded DNA-protein complex, but it is unknown whether the T-DNA integrates into the genome as a single- or double-stranded molecule. In any case, second-strand synthesis cannot start from the 5′ right border end and is therefore unlikely to enhance homologous integration of the 5′ end. Thus, the efficient transfer of the *LEU2* gene with SDM4205 in comparison with SDM4204 must be the result of the neighboring VirD2 protein attached to the 5′ right border.

Deletions of parts of large T-DNAs may occur before T-DNA integration, whereby the left part of the T-DNA is more prone to deletion than the right part. This difference is because the 5′ end is protected against exonucleases by the covalently attached VirD2 protein. In the absence of VirE2, the T-DNA suffers much stronger deletions from the 3′ end (36). Therefore, coating of the T strand with VirE2 also protects the T strand against nucleases. Our finding that the *LEU2* marker is transferred more efficiently if linked to the right border repeat may therefore be due to the stronger preservation of this part of the T-DNA during transfer as a consequence of the presence of VirD2. The sixfold-lower transfer frequency with

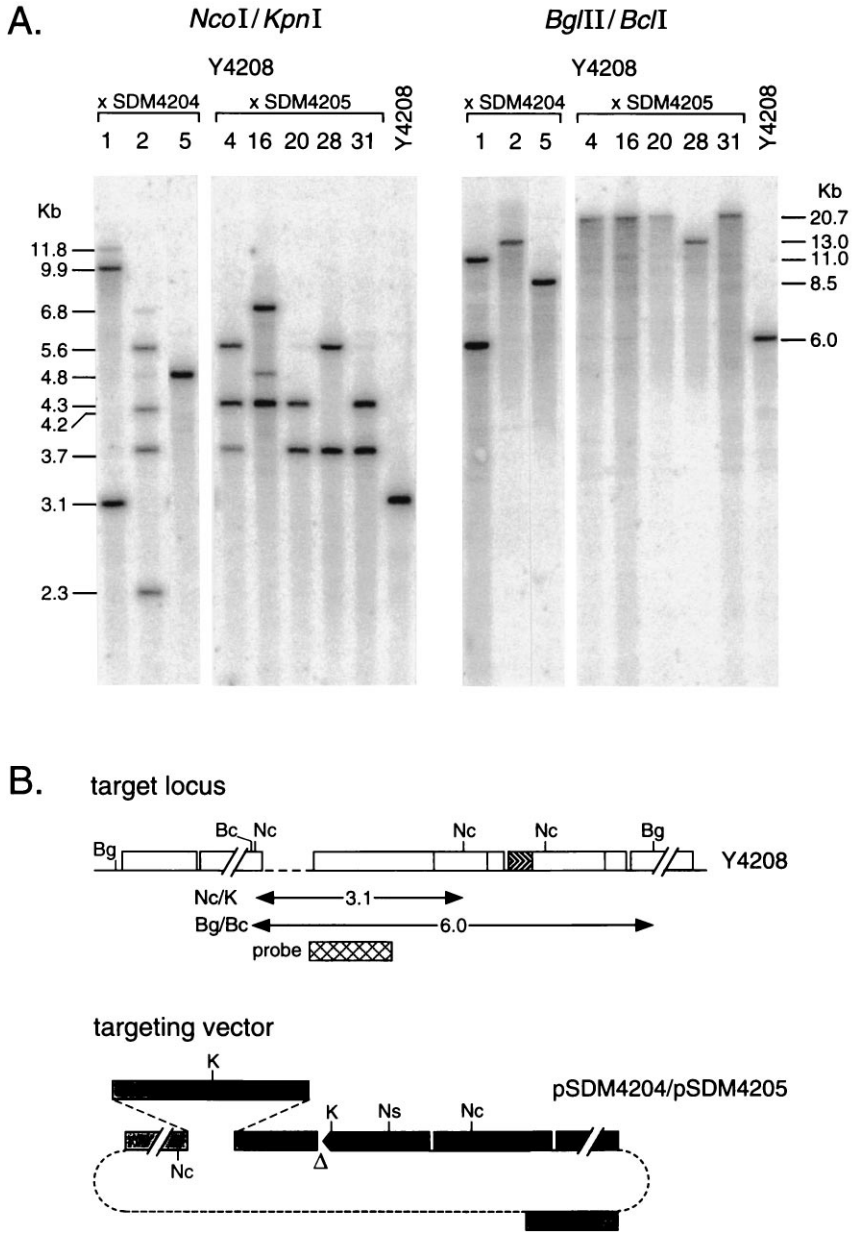


FIG. 4. Southern blot analysis. (A) Yeast genomic DNA from eight chromosomal recombination events was digested with *Nco*I-*Kpn*I and *Bgl*II-*Bcl*I and hybridized with a ^{32}P-labelled 1-kb fragment from the *uidA* coding region. DNA from 4204-1/2 and 4205-4/16/28/31 was partially digested with *Kpn*I. (B) Restriction map of the yeast target strain Y4208. Three restriction sites for *Bcl*I (Bc), *Nco*I (Nc), and *Bgl*II (Bg) were removed in the targeting vector. Filling in of the *Nco*I site resulted in a new *Nsi*I (Ns) site. A *Kpn*I (K) site was located between the homologous DNA and the right T-DNA border. (C) The restriction maps of the chromosomal recombinants from panel A show the expected fragment sizes and the crossover sites between the target locus (open boxes) and the targeting vector (shaded boxes).

SDM4204 would then suggest that significant deletions had occurred in about 85% of the T-DNA population at the left border end. Typically, large deletions are not common at this high frequency during T-DNA integration in plant cells. Moreover, T-DNA truncations and deletions would have affected the homologous integration in *S. cerevisiae* only if they arose before integration. Models proposed for random T-DNA integration in plants favor the appearance of truncations at the left border end as a result of the integration process (12, 25, 26, 42). Finally, the number of border fusion events and the absence of aberrant PCR fragments with primers gus and npt (Fig. 2) revealed that no or little degradation at the T-DNA ends had occurred.

The other possibility is that integration of the right border end is enhanced by the VirD2 protein. Enhanced recombination near the right border end, frequently followed by a second crossover on the same arm, would explain the large number of replacement events with SDM4205. Recently, it was shown that during illegitimate recombination a particular mutation in VirD2 has no negative effect on the T-DNA integration efficiency into the plant genome but leads to the integration of T-DNAs lacking part of the right border end (42). Therefore, it may be that VirD2 somehow is involved in the integration process. Our results are compatible with this hypothesis. Recently, a protein which is covalently bound to the 5′-strand termini of double-strand DNA breaks induced during meiosis

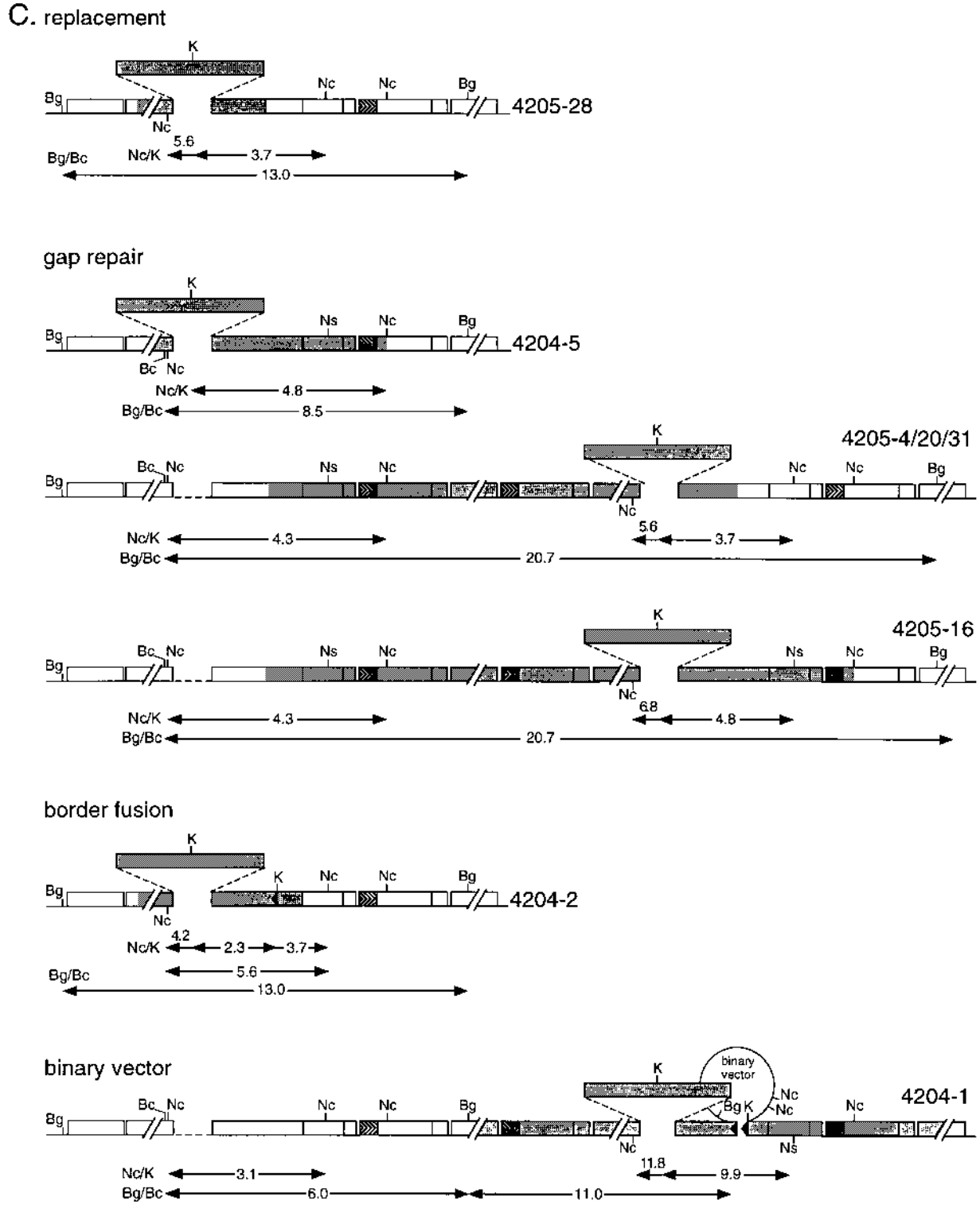


FIG. 4—*Continued.*

was found in *S. cerevisiae* (7, 22, 24). The unknown protein is proposed to be an endonuclease which subsequently prevents the 5′ ends from resection and the formation of 3′ single-stranded ends. Both functions have been shown for VirD2 also, and it might be that VirD2 is recognized and released by a similar mechanism upon homologous integration in the yeast genome.

Irrespective of the role of VirD2, it can be concluded that as well as the host, factors determined by the T-DNA–protein complex also affect the frequency and type of homologous

recombination. For plant gene-targeting experiments, it is recommended to place the right T-DNA border at the targeting vector side which is most important for selection or PCR screening of homologous recombination events.

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