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Yeast Functional Analysis Report

Insertional mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens*

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Abstract

Insertional mutagenesis is a powerful tool for the isolation of novel mutations. The gene delivery system of the bacterium *Agrobacterium tumefaciens*, which mediates transfer not only to plants but also to yeasts and fungi, could be exploited to generate collections of yeasts containing insertional mutations if there were no bias towards particular integration sites, as is the case in plants. To test this, we have analysed a small collection of *Saccharomyces cerevisiae* strains with T-DNA copies integrated in the *S. cerevisiae* genome. The position of 54 of these T-DNAs was determined. The T-DNA showed no clear preference for certain DNA sequences or genomic regions. We have isolated insertions in the coding regions of the genes YGR125w, YDR250c, YGR141w, YGR045c, YPL017c, YGR040w, YDL052c, YJL148w, YCL033c, YFL061w, YJR033c, YDR175c and YLR309c confirming that these genes are non-essential for *S. cerevisiae* haploid growth on minimal medium. Given the advantages of T-DNA, we propose its use as an ideal mobile DNA element for insertional mutagenesis in yeasts. Copyright © 2002 John Wiley & Sons, Ltd.

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Introduction

Insertional mutagenesis has been used extensively in many organisms to link mutant phenotypes with specific genotypic alterations. In yeasts, several different DNA elements have been used for mutagenesis, including transposons (Smith *et al.*, 1996; Ross-Macdonald *et al.*, 1999) and linearized plasmid DNA (Chua *et al.*, 2000). Mutants from such a population can be screened and the disrupted gene identified without the need for cloning or functional complementation. In an ideal screen, such a DNA element integrates randomly in the yeast genome, should remain intact, and should be present in the transformants at a low copy number to simplify later analysis.

Agrobacterium tumefaciens is a Gram-negative bacterium that causes crown gall disease on a wide range of dicotyledonous plant species (for review, see Zhu *et al.*, 2000). An overview of the infection process is shown in Figure 1. Upon wounding of the plant cell, the virulence (*vir*) genes located on the

tumour inducing plasmid (pTi) of *A. tumefaciens* are induced. The T-region of the Ti plasmid has at either end 24 bp imperfect direct repeats known as the left and right borders (LB and RB). The virulence protein VirD2, a relaxase, makes a nick at the bottom strands of the border sequences with the help of the VirD1 protein. This bottom strand of the T-region is released from the plasmid to form the T-strand, presumably via a strand displacement/replication mechanism. Other Vir proteins encode a pore structure for transfer of the T-strand across the bacterial and plant membranes into the plant cell. The VirD2 protein, which has remained covalently attached to the 5' end of the T-strand, ensures its efficient targeting to the plant nucleus. The ssDNA binding protein VirE2 prevents degradation of the T-strand by nucleases. Once in the plant nucleus, the T-strand becomes integrated somewhere in the plant genome and from then on is called T-DNA. Usually the T-DNA remains fairly intact during integration. Transformed cells often have the T-DNA present at a low copy

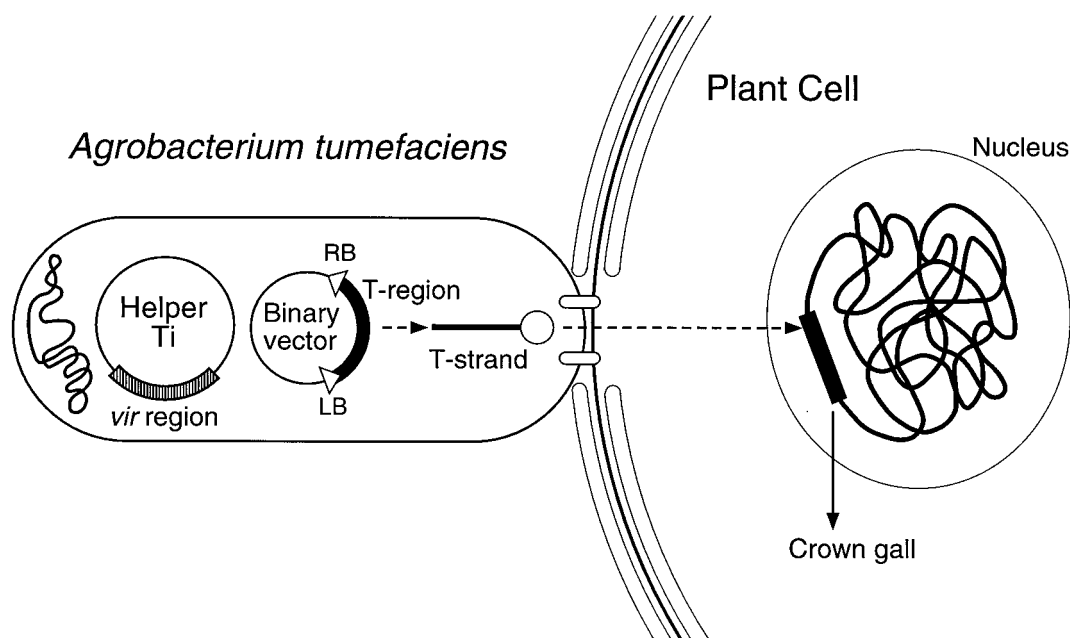


Figure 1. An overview of tumorogenesis

number per transformed cell. In the transformed plant cell, expression of genes located on the T-DNA results in crown gall disease. These genes are not essential for the process of T-DNA transfer and can therefore be replaced by any DNA sequence, which will then be transferred to plants and stably integrated into the plant genome.

We have previously shown that *A. tumefaciens* is also able to transfer its T-DNA to the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* as well as to a wide range of filamentous fungi. T-DNA can therefore be used as a gene vector for these organisms, which is most relevant for species that cannot be otherwise efficiently transformed. In the absence of homology the T-DNA integrates into the yeast chromosomal DNA by non-homologous recombination (NHR) (Bundock *et al.*, 1995, 1999; Bundock and Hooykaas, 1996; De Groot *et al.*, 1999). This suggested that the T-DNA could be used as an effective insertional mutagen for yeasts and fungi. In this report we have mapped the location of 54 T-DNA copies integrated into the genome of *S. cerevisiae* by NHR. The results showed that T-DNA integration events were present in both coding and non-coding DNA and were distributed throughout the *S. cerevisiae* genome, confirming the potential of T-DNA as a promising insertional mutagen in yeasts and fungi.

Materials and methods

Constructs

Plasmid pJJ244 (Jones and Prakash, 1990), containing the *S. cerevisiae* *URA3* ORF on a pUC9 cloning vector, was digested with *Kpn*I and inserted into the *Kpn*I site of binary vector pSDM14 (Offringa *et al.*, 1990) to yield pRAL7207. Plasmid pSDM8000 was constructed by inserting a 1518 bp *Eco*RV–*Pvu*II fragment from plasmid pFA6A (Wach *et al.*, 1994), containing the KanMX gene, into the *Hpa*I site of pSDM14. The constructs pRAL7207 and pSDM8000 were then electroporated to *A. tumefaciens* strains LBA1126 and LBA1119, respectively.

Co-cultivations

Co-cultivations between *A. tumefaciens* strain LBA1126 (pRAL7207) and RSY12 (Schiestl *et al.*, 1991) were carried out as previously described (Bundock *et al.*, 1999). The co-cultivations between LBA1119 (pSDM8000) and YPH250 (Sikorski and Hieter, 1989) were performed in a slightly different way. These were done for 9 days at 20°C and selection was carried out on YPAD medium containing 200 µg/ml G418 (Life Technologies/Gibco BRL).

Rescue of the *S. cerevisiae* sequences linked to the T-DNA RB

Non-radioactive Southern blotting was carried out as described (Neuhaus-Url and Neuhaus, 1993). 2 µg total yeast DNA was digested using *EcoRI* or *SacI*, run on a 0.8% TBE gel and blotted to a nylon membrane. Two different probes were made. A 1.1 kb *HindIII* fragment containing the *S. cerevisiae URA3* gene was labelled using the DIG DNA Labelling Kit (Boehringer-Mannheim). A 792 bp DIG-labelled PCR fragment consisting of the KanMX ORF was made using the primers KanMXp1 (5'-AGACTC ACGTTTCGAGGCC) and KanMXp2 (5'-TCACC GAGGCAGTTCCATAG) and plasmid pFA6A as a template (Wach *et al.*, 1994). The *URA3* and KanMX probes were used on the blots containing DNA from the transformants generated using LBA1126 (pRAL7207) or LBA1119 (pSDM8000), respectively. Rescue of the yeast DNA linked to the RB of T-DNAs derived from pRAL7207 was done as previously described (Bundock *et al.*, 1996), using the restriction enzymes *EcoRI* or *SacI*, depending on which restriction enzyme gave the smallest band on the DNA blot. The primer 7207RB (5'-CAGTTATTACCCGGGAAT) was used for sequencing. Vectorette PCR (Riley *et al.*, 1990) with adaptations (<http://www-genome.stanford.edu/group/botlab/protocols/vectorette.html>) was done to rescue the yeast sequences linked to the RB of T-DNAs derived from pSDM8000. Chromosomal DNA was digested with *EcoRI* and used in a ligation with an *EcoRI* vectorette linker. PCR was done on this ligation mix using primers p224 (5'-CGAATCGTAACCGTTTCGTACGAGAATCGCT) and a T-DNA-specific primer, KanMXp2. The PCR products were then cloned in pGEM T Easy (Promega) and sequenced using the nested primer pKanMXp5 (5'-TCACATCATGCCCTGAGCTGC).

Results

The *A. tumefaciens* strains used in this study contain a so-called 'helper' Ti plasmid from which the T-region is deleted (Figure 1). The T-region is present *in trans* in *Agrobacterium* on a *A. tumefaciens*-*E. coli* shuttle plasmid called the binary vector (pRAL7207 or pSDM8000). The T-strand produced from the binary vector pRAL7207 carries the *S. cerevisiae URA3* gene, the *bla* gene encoding resistance to the antibiotic carbenicillin and the *ori*

sequences required for replication in *E. coli*. Plasmid pRAL7207 was electroporated to the *A. tumefaciens* strain LBA1126. Co-cultivations were carried out with LBA1126 (pRAL7207) and *S. cerevisiae* strain RSY12 (Schiestl *et al.*, 1991). Selection for transformants was done on medium lacking uracil. In RSY12 the *URA3* locus has been deleted. The T-DNA therefore carries no DNA homology with the genome of RSY12 and can only integrate into the *S. cerevisiae* genome by non-homologous recombination, as was previously observed (Bundock *et al.*, 1996). The T-strand of binary vector pSDM8000 also lacks homology with the genome of *S. cerevisiae*. It carries the KanMX marker flanked by heterologous sequences. This marker allows selection of transgenic yeasts resistant against G418. The binary vector pSDM8000 was electroporated to *A. tumefaciens* strain LBA1119. The sequences of the T-DNAs from pRAL7207 and pSDM8000 were used in a BLAST search to detect any large regions of homology between the T-DNA and the yeast genome. Besides the *URA3* gene on the T-DNA of pRAL7207, which was transferred to RSY12 (*URA3* deletion), no large regions of shared homology were found. Co-cultivations were carried out between LBA1119 (pSDM8000) and *S. cerevisiae* strain YPH250. In this case selection for transformants was carried out on medium containing G418. The results of these co-cultivations are shown in Table 1. The yeast strains RSY12 and YPH250 were used in this study. Integration of T-DNA in these strains by non-homologous recombination (NHR) was not very efficient. In later experiments we utilized the strain JKM115 (Moore and Haber, 1996), which gave up to 10³ transformants after a 9 day co-cultivation with *Agrobacterium*. This demonstrates that the efficiency of

Table 1. Co-cultivations between *A. tumefaciens* and *S. cerevisiae*

Co-cultivation	Transformants	Frequency ³
LBA1126 (pRAL7207) × RSY12 ¹	12	3.5 × 10 ⁻⁸
LBA1119 (pSDM8000) × YPH250 ²	58	1.6 × 10 ⁻⁷

The data represents the average of at least three independent experiments.

¹Co-cultivations were done for 3 days.

²Co-cultivations were done for 9 days.

³Expressed as the number of *S. cerevisiae* colonies growing on selective medium divided by the total number of *S. cerevisiae* cells present after the co-cultivation period.

NHR can vary greatly between different yeast backgrounds.

Total yeast DNA from the *S. cerevisiae* colonies that grew on the selection medium was digested with *EcoRI* and used for a DNA blot. The *URA3* or *KanMX* genes were used as probes. These probes detect the DNA fragments encompassing the T-DNA up to the RB and the yeast chromosomal sequences linked to the T-DNA right border. In the majority of transformants only a single band was detected on the blot, suggesting a single integrated T-DNA copy (data not shown). In a small number of transformants (1–5%) two bands were present on the blot. Further analysis of these strains showed that they often contained two T-DNA copies present at a single locus in an inverted repeat structure. The yeast chromosomal sequences flanking the

pRAL7207 T-DNA insertions and the pSDM8000 T-DNA insertions were isolated using either plasmid rescue or vectorette PCR, respectively. The yeast sequences linked to the T-DNA right borders were used in a BLAST search of the complete *S. cerevisiae* genome (<http://www-genome.stanford.edu/SGD>). We were able to map the insertion points of 54 T-DNAs precisely (Figure 2) and the data is summarized in Table 2.

Based upon sequence data only, three T-DNA insertions could not be precisely mapped. One strain contained the T-DNA integrated into a highly conserved subtelomeric ORF, as found for strain 11, but in this case an identical sequence is found at the end of 10 different chromosomes. Two T-DNA insertions mapped to a yeast repetitive element, the Ty1 retrotransposon. More data on these strains is

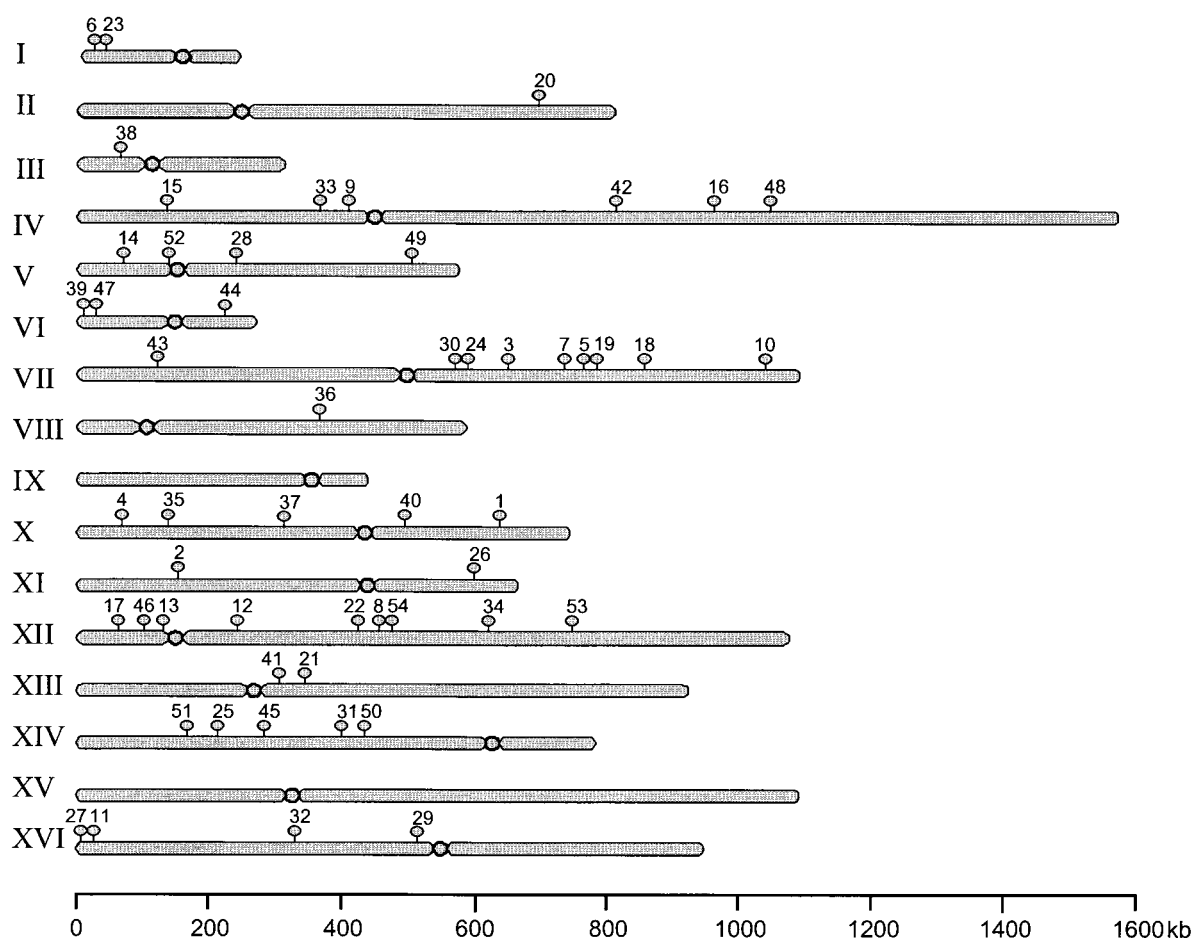


Figure 2. Distribution of T-DNA insertions over the 16 chromosomes of *S. cerevisiae*. The chromosomes are drawn to scale, including centromeres, and the positions of the T-DNA insertions are indicated

necessary to determine on which chromosome the insert is present. The T-DNA in strain 8 is located in a rDNA repeat. The number of rDNA repeats is variable, and in some strains they can make up to half of chromosome XII (Olsen, 1991). The T-DNA is therefore positioned in Figure 2 in the region of chromosome XII reported in the *S. cerevisiae* database that contains the rDNA repeats.

T-DNA copies were found distributed over most chromosomes. The total number of T-DNA insertions found in each chromosome varied, from nine T-DNA insertions in chromosome VII to only one insertion in several of the other chromosomes. If T-DNA integration was completely random, we would expect the distribution of the T-DNA insertions along the chromosomes, and indeed throughout the genome, to be fairly uniform. We cannot conclude much from the chromosomes with only one or two insertions, but chromosomes VII and XII contain enough insertions to be able to address this question. The pattern of T-DNA insertions in chromosome VII of RSY12 seems exceptional. Eight of the nine T-DNA insertions in this chromosome were found distributed along the right chromosome arm, and in some cases clustered together (24 and 30; 7, 5 and 19). A more detailed examination of this region revealed that many of the T-DNA copies had integrated very close to yeast retrotransposons, the Ty elements. Ty elements are one of the few sources of repetitive DNA in the yeast genome. They are relatively rare (53 copies) and are subdivided into five different classes, Ty1–Ty5. Of these, Ty1 elements are the most abundant. Surprisingly, Ty elements are also not distributed evenly along chromosome VII. All six Ty elements on this large chromosome (1091 kb) are also all present on the right chromosome arm (Hani *et al.*, 1998). Due to this association between T-DNA insertions and retrotransposons, we then examined the proximity of T-DNA copies and Ty elements in other chromosomes. However, in none of the remaining chromosomes was an association between Ty elements and the T-DNA insertions apparent. Thus, the chromatin structure of the right arm of chromosome VII may favour the efficient integration of T-DNA and Ty elements. We have already observed that the efficiency of T-DNA integration in the yeast genome by NHR depends strongly on the genetic background. The distribution may therefore also be partially dependent upon the genetic background of the yeast strain used. In support of this, of the 10 T-DNA insertions studied

in *S. cerevisiae* strain YPH250, none mapped to this region of chromosome VII.

Table 2 summarizes the data we obtained for each T-DNA insertion. On average, a typical yeast gene consists of a 309 bp upstream element, a 1450 bp ORF and a 163 bp downstream element (Dujon, 1996). Of the total 54 T-DNA insertions, 13 were located in an upstream element (24%), 14 were found in ORFs (26%), three were located in downstream elements (6%) and 22 were located in intergenic regions (41%). The remaining two strains (8 and 52) contained a T-DNA integrated into the genes for rDNA and t-RNA-Glu, respectively. Two strains (37 and 43) contained the T-DNA inserted in an area in which the upstream and downstream regions of two divergently transcribed genes overlap. Therefore we conclude that T-DNA integration in *S. cerevisiae* does not show any clear preference for particular regions or genes. Additionally, the orientation of T-DNA copies integrated into both coding and non-coding regions of the yeast genome was variable. Thus, T-DNA seems a promising element for use as an insertional mutagen in yeasts.

Discussion

We describe the distribution of 54 T-DNA insertions throughout the genome of the yeast *Saccharomyces cerevisiae*. Previous studies examining the distribution of T-DNA throughout the plant genome have concluded that T-DNA integration at the genome level appears to be random (Ambros *et al.*, 1986; Chyi *et al.*, 1986; Wallroth *et al.*, 1986; Thomas *et al.*, 1994; Tinland, 1996). Studying the integration pattern of the T-DNA insertions generated in this study, we can also conclude that T-DNA integration in *S. cerevisiae* shows no strong preference for particular DNA sequences or regions of the genome. At the DNA level, T-DNA insertions were found in intergenic, promoter, coding and downstream regions. T-DNA copies have also been found to be distributed throughout the genome of *Arabidopsis thaliana* and to have no preference for certain regions of genes (Azpiroz-Leehan and Feldman, 1997). T-DNA is transferred to the recipient cell as a DNA–protein complex. It is therefore conceivable that the VirD2 protein, covalently linked to the T-DNA 5' end, and the VirE2 protein, a non-specific ssDNA binding protein which coats the T-DNA along its length,

Table 2. The positions of randomly integrated T-DNA copies in the *S. cerevisiae* genome

Strain	Chromosome	Insertion point ¹	Orientation	Description
1	X	643888	+	Promoter
2	XI	152085	+	Intergenic
3	VII	649057	+	Intergenic
4	X	68739	-	Promoter
5	VII	762216	-	Downstream
6	I	20900	+	Intergenic
7	VII	745285	+	ORF YGR125w
8	XII	455390/464527	+	rDNA
9	IV	410258	+	Intergenic
10	VII	1039487	-	Intergenic
11	XVI	12179	-	Subtelomeric ORF
12	XII	241736	+	Intergenic
13	XII	143086	+	Promoter
14	V	69286	-	Intergenic
15	IV	132782	-	Intergenic
16	IV	960237	+	ORF YDR250c
17	XII	83966	-	Intergenic
18	VII	856942	-	Intergenic
19	VII	771886	+	ORF YGR141w
20	II	697483	+	Promoter
21	XIII	305993	+	Intergenic
22	XII	448563	+	Intergenic
23	I	28768	-	Intergenic
24	VII	584187	+	ORF YGR045c
25	XIV	211620	-	Downstream
26	XI	599386	-	Promoter
27	XVI	6955	+	Promoter
28	V	242313	+	Intergenic
29	XVI	520200	-	ORF YPL017c
30	VII	575792	-	ORF YGR040w
31	XIV	408146	+	Promoter
32	XVI	329068	-	Intergenic
33	IV	362716	-	ORF YDL052c
34	XII	625121	+	Intergenic
35	X	140189	+	ORF YJL148w
36	VIII	367974	-	Intergenic
37	X	313752	-	Promoter/downstream
38	III	63118	-	ORF YCL033c
39	VI	9526	+	ORF YFL061w
40	X	496053	+	ORF YJR033c
41	XIII	347009	+	Intergenic
42	IV	813507	-	ORF YDR175c
43	VII	123427	-	Promoter/downstream

Table 2. Continued

Strain	Chromosome	Insertion point [†]	Orientation	Description
44	VI	224082		Downstream
45	XIV	287780	+	Promoter
46	XII	108079		Intergenic
47	VI	23040		Promoter
48	IV	1055118	+	Promoter
49	V	517730	+	Intergenic
50	XIV	441139	+	Intergenic
51	XIV	185313	+	Intergenic
52	V	177107	+	t-RNA-Glu
53	XII	751534	+	ORF YLR309c
54	XII	491862		Promoter

Strains 1–44 resulted from co-cultivations between LBA1126 (pRAL7207) and RSY12. Strains 45–54 were generated after co-cultivation of LBA1119 (pSDM8000) with YPH250. The insertion point represents the nucleotide on each yeast chromosome that was found linked to the T-DNA right border. + and – represent the two possible orientations of the integrated T-DNA copies. The precise position of the T-DNA copy in strain 8 cannot be determined because it is located in a rDNA repeat. The T-DNA copies in strains 37 and 43 are located in the promoter of an ORF encoded on the Watson strand and in a region of the Crick strand which serves as a promoter and terminator for two different genes. When a strain contained a T-DNA insertion in an ORF, the *Saccharomyces* Genome Database was checked to confirm that disruption of the ORF was not lethal in haploid yeast. In each case the ORFs were not essential for haploid yeast growth.

may play a role in the integration process. Based on our data, these proteins do not seem to provide any obvious bias for the pattern of T-DNA integration. We also did not observe any role for these proteins when studying integration of T-DNA into the yeast genome via homologous recombination (Bundock *et al.*, 1995). Most transformants contained a single T-DNA copy and sequencing showed that the T-DNA ends remained intact (data not shown). Previous studies in which the yeast DNA flanking both the T-DNA ends was rescued showed that in only two out of the 11 T-DNA inserts studied, microhomology was present between the T-DNA ends and the yeast integration site (Bundock *et al.*, 1996). The presence of microhomology was associated with small (5 bps and 18 bps) deletions at the right borders. Integration of non-homologous DNA fragments has also been utilized to generate mutated yeast populations, but in these cases microhomology was always observed between the genomic DNA and the end of the integrated cassette, which could bias the integration pattern (Chua *et al.*, 2000).

In the transformants generated using yeast strain RSY12, we often found that the T-DNA had integrated into the right arm of chromosome VII. The Ty elements were also found to be clustered on this chromosome arm. It has been reported that double-strand breaks (DSBs) in the yeast genome can also be repaired utilizing retrotransposon cDNA produced during transposition (Teng *et al.*, 1996; Moore and Haber, 1996b). DSBs in the plant genome can also be repaired utilizing endogenous plant retrotransposons or T-DNA (Salomon and Puchta, 1998). This suggests that both T-DNA and retrotransposon-derived cDNAs may be used to patch up a DNA lesion. Such a mechanism of DSB repair may occur efficiently on the right arm of chromosome VII, thus perhaps partially explaining the tight association of Ty elements and T-DNA. Alternatively, this region of chromosome VII may be 'fragile' in strain RSY12 and be prone to frequent DNA damage.

A. tumefaciens-mediated transformation of plant cells has become the most popular method for the introduction of transgenes into a wide variety of plant species. Its ease of use, efficiency and simple T-DNA integration patterns simplify the eventual analysis of transformants. The current work indicates that this useful bacterium can now also be similarly utilized for the mutagenesis of the yeast genome.

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