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Genetic aspects of targeted insertion mutagenesis in yeasts

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Abstract

Targeted insertion mutagenesis is a main molecular tool of yeast science initially applied in *Saccharomyces cerevisiae*. The method was extended to fission yeast *Schizosaccharomyces pombe* and to "non-conventional" yeast species, which show specific properties of special interest to both basic and applied research. Consequently, the behaviour of such non-*Saccharomyces* yeasts is reviewed against the background of the knowledge of targeted insertion mutagenesis in *S. cerevisiae*. Data of homologous integration efficiencies obtained with circular, ends-in or ends-out vectors in several yeasts are compared. We follow details of targeted insertion mutagenesis in order to recognize possible rate-limiting steps. The route of the vector to the target and possible mechanisms of its integration into chromosomal genes are considered. Specific features of some yeast species are discussed. In addition, similar approaches based on homologous recombination that have been established for the mitochondrial genome of *S. cerevisiae* are described.

Keywords: Yeasts; Non-*Saccharomyces* yeasts; Non-conventional yeasts; Targeted insertion mutagenesis; Gene disruption; Gene replacement; Biolistic bombardment

Contents

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1. Introduction

The paradigm for a yeast is *Saccharomyces cerevisiae*, the baker's yeast, that has already been used for studies on carbohydrate metabolism since about 1830 [1]. It is a matter of course that meanwhile *S. cerevisiae* provides a eukaryotic model system very well suited for the examination of basic and applied biological aspects. In addition to *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* is widely used as a model organism as well, mainly for studies on the cell cycle and its regulation [2,3]. The knowledge of the complete genomic DNA sequences of both "conventional" yeasts provides an excellent molecular background for studies by reverse genetics [4].

Among the more than 600 yeast species that have been described until now, there is a number of "non-conventional" yeasts with specific properties of special interest to both basic and applied research [5–11]. Some of these yeasts are of importance because of their physiological properties differing from *S. cerevisiae*. We will support this supposition by the following examples, without laying claim to completeness: *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* [12,13] are osmotolerant yeasts and are used for both basic and applied research on this characteristic. *Kluyveromyces lactis* [14,15] may ferment lactose, the main sugar of whey. This "milk yeast" is Crabtree-negative, a property that possibly makes it more suitable as a host for the production of heterologous proteins than *S. cerevisiae* [16]. Effective heterologous protein expression systems have been developed based on the methylotrophic yeasts *Hansenula polymorpha* [17–21] and *Pichia pastoris* [17,22,23]. These species and the likewise methylotrophic yeast *Pichia methanolica* [24] were used for the investigation of peroxisome biogenesis [25,26] and other specific genetic and physiological properties. *Pichia stipitis* may ferment xylose [27–30] and possesses a particular hypoxia-inducible gene expression system with similarity to the mammalian HIF-1 system [31]. *Schwanniomyces occidentalis* [32,33] can efficiently degrade starch, due to the inducible secretion of both α-amylase and glucoamylase. *Yarrowia lipolytica* [34] and *Candida maltosa* [35] are effective *n*-alkane utilizers. Particularly, *C. maltosa* was used to investigate the alkane oxygenising enzyme cytochrome-P450 [36,37]. The anamorphic yeast *Arxula adeninivorans* [38] has also strong amylolytic properties. Moreover, it has extremely versatile utilization properties and is both osmo- and thermotolerant and shows an interesting temperature-dependent dimorphism [39]. The anamorphic species *Candida albicans* [40–43], *Candida dubliniensis*, *Candida glabrata* and *Candida tropicalis* are facultative pathogenes of increasing importance [44] and, as a consequence, in the center of interest of medical microbiology.

Genomes of some of the non-conventional yeasts are currently at least partly sequenced, which will improve the conditions for application of reverse genetics in the future [45–49].

A main tool of yeast science is targeted insertion mutagenesis. It is induced by in vivo insertion of suitable vector DNA into a homologous locus of a chromosome, after transformation of the cells. This powerful method was used for the first time over 20 years ago in *S. cerevisiae* [50,51] and has been permanently improved in the meantime. Recently, targeted insertion mutagenesis in *S. cerevisiae* was demonstrated using simply pairs of oligonucleotides homologous to the flanks of the targeted DNA sequence [52].

Targeted insertion mutagenesis has been applied in a number of other yeast species with several modifications and with sometimes different consequences and changing success. In many non-*Saccharomyces* yeasts, such as in higher eukaryotes including man, targeted insertion mutagenesis is hampered by a strong bias against homologous integration. The desired mutants have to be detected among a more or less large number of transformants by an elaborate screening process. Recently, the method of gene targeting has been optimized for application in higher eukaryotes [53,54].

The aim of this review is to summarize the current knowledge of the behaviour of some non-*Saccharomyces* yeasts against the background of the extended knowledge of targeted insertion mutagenesis in *S. cerevisiae*. In the first part, principles of targeted insertion mutagenesis in *S. cerevisiae* will be shortly summarized and the different vector types will be classified to facilitate further reviewing. The behaviour of different vector types will then be compared in the different recipient yeast species. These data will be discussed from the point of view of possible rate-limiting steps of both efficiencies of transformation and homologous integration. Current knowledge on the molecular mechanisms during the integration of different vector types in *S. cerevisiae* will be taken into consideration in connection with the results of targeted insertion mutagenesis that were obtained with other yeasts species.

2. Strategies

2.1. Saccharomyces cerevisiae

Details of transformation, vector systems and their different application possibilities are summarized in a number of excellent reviews [55–61].

The history of targeted insertion mutagenesis in yeast started with the first successful integrative transformation of *S. cerevisiae* [50]. Hinnen et al. used a circular vector (pYe*leu* 10) containing bacterial sequences, the *S. cerevisiae LEU2* gene, but no autonomous replication

sequence (ARS). In a *leu2* recipient, 88% of the transformants showed a homologous integration of the vector DNA. In 30 out of 37 homologous integrants, a duplication of the *LEU2* region was observed, indicating an integration of the entire vector. These transformants, formed by "targeted additive integration", were of limited stability. The Leu$^-$ phenotype segregated with a frequency between 1% and 2%, indicating frequent excision of the vector DNA by homologous intrachromosomal mitotic recombination. Further homologous integrants were stable, but contained no vector DNA. They had originated, obviously, by homologous integration of only the *LEU2* region of the vector, which means by substitution of the mutated allele for the wild-type allele, probably by gene conversion.

This integrative vector type has a low transformation efficiency in comparison with replicative vectors, that were described to be as high as 2×10^7 in *S. cerevisiae* [58]. This type of circularly closed integrative vectors will be designated in this review generally as *circular vectors*. Scherer and Davis [62] demonstrated that it was useful in *S. cerevisiae* for targeted replacement of chromosomal segments and Shortle et al. [63] showed that it was useful for gene disruption in case the vector contains an internal fragment of the target gene. A general scheme of circular vector organization, integration and excision is presented in Fig. 1. Selection can be performed either by the p:target sequence itself (as in the case of Hinnen's experiments), or by a distinct selection marker that is apart from p:target (as shown in Fig. 1).

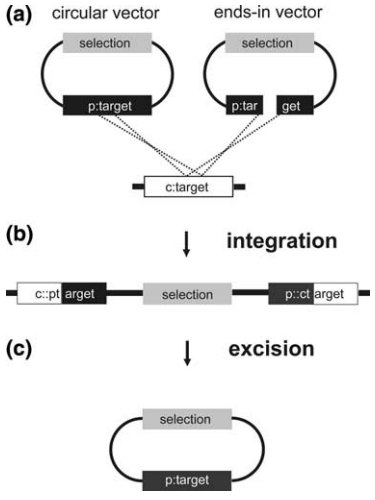

Fig. 1. Structure and integration modus of circular and ends-in vectors. (a) Ends-in vectors are linearized circular vectors with a gap in the p:target region. (b) Both vector types integrate additively. Duplication of the target occurs if p:target consists of an intact gene. The disruption of the gene leads to a mutation if a 5′ and 3′ truncated copy of the target reading frame is used. (c) The vector molecule can be excised from the chromosome by mitotic recombination between both homologous target regions. p:target – region of the vector with homology to the target gene; c:target – chromosomal target region; selection – selection marker.

Orr-Weaver et al. [64,65] discovered that linearization of a circular vector within the p:target region tremendously enhances the efficiency of transformation and integration in *S. cerevisiae*. The yield of transformants increased up to three orders of magnitude. The ends of the linearized vectors (generated either by a restriction cut or by deletion of an internal DNA sequence, Fig. 1(a)) strongly increased the recombination frequencies. Because of the orientation of the homologous p:target regions, Hastings et al. [66] designated these linearized molecules as *ends-in vectors.* After homologous pairing with the c:target region they form a gap (Fig. 1(a)). These gaps are repaired using the homologous c:target DNA as a template. These observations led to the meanwhile generally accepted model of double-strand break repair of recombination in yeast [67–69]. Ends-in vectors were demonstrated to integrate in *S. cerevisiae* very frequently in multiple tandem repeats by sequential integration of discrete vector molecules [70]. The mitotic stability of integrated ends-in plasmids is comparable to that of integrated circular plasmids.

In 1983, Rothstein described the method of one-step gene disruption in *S. cerevisiae* [51,57]. This method is based on the integration of a linear DNA fragment into a target region (Fig. 2). The target DNA is replaced by vector DNA. The vector molecule consists of a central region that is, in most cases, provided with a selection marker, which often shares no homology with the recipient DNA sequences. The central region is flanked by two short DNA regions, homologous to the borders of the target region. We will designate this kind of molecules, according to Hastings et al. [66], as *ends-out vectors* (Fig. 2). Just as ends-in molecules they are linear, however, they exhibit different arrangements of their p:target sequences. These different topologies cause different configurations of homologous pairing of both vector types with the target DNA (Figs. 1 and 2).

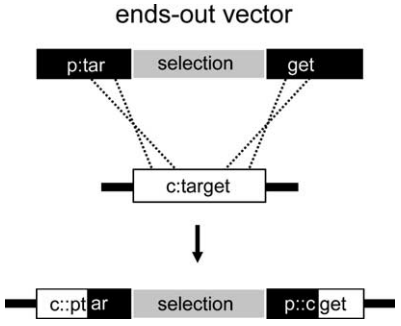

Fig. 2. Structure and integration modus of ends-out vectors. The vector contains a central selection cassette between two fragments of the target gene. These fragments are homologous to the 3′ and the 5′ border regions of the desired replacement. The target region was replaced by the integrated vector. p:target – (black bars) region of the vector with homology to the target gene; c:target – (white bars) chromosomal target region; selection – (light grey bars) selection marker.

Pairing of ends-out vectors with the target region is not connected with the formation of a double-strand gap, as it is the case for ends-in vectors.

Ends-out vectors have mainly been used for recent targeted insertion mutagenesis in *S. cerevisiae*. Transformation efficiencies are, in most cases, low and comparable to those obtained with circular vectors. By means of foreign carrier-DNA these efficiencies can be remarkably enhanced from about 2 up to 2000 transformants μg DNA^{-1} [71].

In contrast to circular and ends-in vectors, stability of the vector substitutes is high, because their integration is not accompanied by the formation of duplicated homologous regions that may serve as a substrate for subsequent excision.

There are some variations in the ends-out vector transformation, such as utilization of co-transformation events or marker recycling (see below). Rudolph et al. [72] described integrative transformation of yeast cells with both an ends-out vector and an episomal vector carrying the selection marker. In this approach, the transformants have to be screened for the co-transformed and integrated ends-out vector molecule.

Construction of ends-out vectors was remarkably facilitated by the application of the PCR technique in *S. cerevisiae* [73–76] and *S. pombe* [77,78]. By this method, linear plasmids with short or long flanking homologous regions can be obtained without subcloning of the vector components.

Selection markers of ends-out vectors can be used repeatedly by marker recycling. The process is made possible by short direct repeats flanking the central selection marker. After integration, the marker is excised by mitotic recombination between these repeats, as demonstrated in *S. cerevisiae* [79]. Then, the marker can be used again for selection of transformants in the same recipient.

Combinations of site-specific recombinases and their target sequences can achieve high frequency of excision. Such systems are, for instance, the lox/cre system of phage P1 [80,81] or the FRT/FLP system of the 2μ plasmid of *S. cerevisiae* [82].

2.2. Non-Saccharomyces yeasts

The transformation efficiencies specified in this review were obtained by different laboratories, with different transformation methods and different yeast strains. Therefore, an exact comparison of one transformation efficiency with the other is almost impossible. However, we disregard marginal differences and consider only distinct differences of one or more orders of magnitude as they are known for *S. cerevisiae*, depending on the vector type used [83].

Most of the results published on integrative transformation have been achieved by experiments with *Hansenula polymorpha* or *Pichia pastoris*. We have not considered all these results, because, in most cases, the aim of these transformations was not targeted insertion mutagenesis but the establishment of hosts for the stable and effective expression of heterologous gene products (see [21,23] for recent reviews).

2.2.1. Circular and ends-in vectors

In Table 1 we have listed results that were obtained from transformation experiments using circular or ends-in vectors. In most of the yeasts, transformation with circular vectors results in the occurrence of transformants with a low efficiency, ranging from approximately 1–50 colonies per μg DNA. In *Candida oleophila* and *Pfaffia rhodozyma* no transformants could be isolated after using this type of vector [84,85]. However, some vector/recipient combinations led to remarkably higher efficiencies of transformation if a circular vector was used. In *Pichia pastoris* and *Candida tropicalis* Cregg et al. [86] and Haas et al. [87] obtained small colonies with a high frequency. The instabilities of the transformants support the supposition that the applied vectors pYA4 and pCU1 contained unknown sequences that could function as an ARS in *P. pastoris* or *C. tropicalis*. Such vectors are generally not useful for targeted mutagenesis, because it is difficult to recover the infrequently occurring integrants amongst the majority of episomal transformants.

Faber et al. [88] observed high efficiencies with circular vectors carrying the *LEU2* gene of *S. cerevisiae* as a selection marker or with *H. polymorpha* sequences homologous to *AOX* or *AMO* of *H. polymorpha*. The transformants were mitotically unstable. This result is explained by the earlier described ARS activity of the *ScLEU2* region in *H. polymorpha* [89]. Among 850 transformants no homologous integrant was found.

Furthermore, Roggenkamp et al. [90] and Gatzke et al. [91] obtained unstable transformants using the *ScURA3* region as a selection marker.

Sequences without ARS activity led to low transformation efficiencies with circular vectors in *H. polymorpha* [91,92]. However, the drawback here is the low frequency of homologous integration in this yeast (see below).

In many cases, the integration modus of circular vectors was not specified. In *C. albicans*, *S. pombe* and *Y. lipolytica* mainly homologous integration was found [93–95]. In *H. polymorpha* two studies using vectors without ARS activity describe striking results: in one case, 50% of homologous integration was reported [92], whereas in the other case no homologous integrants could be found [91] (Table 1).

In comparison with circular vectors, experiments with linearized ends-in vectors resulted in many cases in a distinct increase of transformation efficiencies (Table 1). A tremendous increase was observed in *C. glabrata* [96],

S. pombe [97] and *Y. lipolytica* [98], whereas a less drastic effect was found in *C. oleophila* [84] and *P. stipitis* [99]. In *C. tropicalis*, stable transformation was only observed with ends-in vectors, but not with circular vectors [87]. The observation of enhanced transformation efficiencies with linearized plasmids suggests participation of double-strand repair systems in the corresponding host yeasts, comparable to that in *S. cerevisiae* [68].

In many cases, homologous integration can be observed with ends-in vectors (Table 1), although Chand-Goyal et al. [100] observed mainly ectopic integration in *C. oleophila*. However, the authors used the *HIS5* gene from *S. cerevisiae* for targeting the *HIS5* gene in *C. oleophila*. The DNA homology between both genes is expected to be comparatively low. Yehuda et al. [84] used an *EXG5* (exoglucanase) sequence of *C. oleophila* as p:target and found high frequency of integration into the *EXG5* gene.

Cormack and Falkow [96] investigated the integration of ends-in vectors in *C. glabrata* in a more detailed way. They used a bacterial Tn903 sequence both as p:target and c:target and observed a correlation between the length of the p:target and the efficiency of homologous integration. A homologous region of more than 200 bp resulted in more than 90% of homologous integration.

Investigations on the mechanism of integration have been performed in *S. pombe*, where mainly homologous integration of ends-in vectors was observed [94,97]. Homologous integration may proceed as in *S. cerevisiae*, either by homologous additive integration or by substitution of p:target for c:target by gene conversion. Such substitutive integration of p:target could be desirable, because the resulting mutant strains lack homologous substrates for excision and the integrated sequences should be stable. However, this benefit can only be utilized if p:target and the selection marker are identical.

In contrast to the situation in *S. cerevisiae*, Grimm and Kohli [94] observed mainly gene conversion in *S. pombe*. On the other hand, Keeney and Boeke [97] found that the majority of mutants showed targeted additive integration and only 20% of the mutants was the result of gene conversion.

A further method for targeted mutagenesis is derived from restriction-enzyme-mediated integration (REMI) [101], that was established to be a mutagenesis system in fungi [102,103]. In *P. stipitis*, the transformation efficiency is remarkably enhanced by using ends-in vectors and REMI [104]. Also, the efficiency of the homologous integration can be enhanced in dependence on the c:target region and the restriction enzyme [99].

2.2.2. Ends-out vectors

In most cases, ends-out vectors were used for targeted mutagenesis not only in *Saccharomyces cerevisiae*, but also in various non-*Saccharomyces* yeasts. Unfortunately, most studies contain no quantitative information about either the efficiencies of the transformation or the ratio of homologous versus ectopic integration. A collection of representative data is given in Table 2.

Transformation of different yeasts with ends-out vectors resulted usually in low transformation efficiencies. Surprisingly, in *C. dubliensis* high efficiencies of transformation were observed [42]. These high rates were obviously not due to ARS properties of a vector sequence, because in more than 90% of the transformants vector integration was detected. It is worth noting that the transformation was performed by the electroporation method.

High frequencies were also found in other yeasts, where stable and unstable transformants were observed simultaneously (Table 2). Instability perhaps points to an ARS activity on the vector sequence of psuc1-D4 in *S. pombe* [105] or the vector construct used in *P. methanolica* [106].

With respect to the relation between homologous and ectopic integration, yeast species behaved differently (Table 2). Similar to *S. cerevisiae* [101], *C. dubliniensis*, *C. tropicalis* and *C. albicans* showed a high rate of homologous integration if long p:target regions were used. Large p:target sequences enhanced the frequency of homologous integration in *C. albicans* [107–110]. If short p:targets of about 60 bp length were used, only infrequent homologous integration was observed in this yeast [111,112].

Moreover, the frequency depended on specific alleles that were targets of insertion mutagenesis. For example, Wilson et al. [111] intended to disrupt both *ARG5* alleles in the diploid *C. albicans* by successive transformations using cassettes with the selection markers *CaHIS1* and *CaURA3*. As expected, disruptions of one of the two alleles in a homozygous *ARG5 ARG5* recipient occurred with similar frequencies (Table 2). However, further disruption of the second wild-type allele in heterozygous *ARG5 arg5::HIS1* disruptants was strongly biased. This second transformation with the *URA3* cassette resulted mostly in the replacement of the *arg5::HIS1* allele by the *URA3* cassette. While 21 of 27 transformants were of this heterozygous *ARG5 arg5::URA3* genotype, only one transformant contained the heterozygous *arg5::URA3 arg5::HIS1* double disruption.

Unlike the *Candida* species mentioned above, data from other yeasts confirm a high level of ectopic integration, even if the vectors carried relatively long regions of homology to the target as in *K. lactis*, *P. methanolica*, *P. pastoris*, *P. stipitis* or *Z. bailii* (Table 2).

In case of the *PIM1* gene of *P. pastoris*, Cosano et al. [113] found that only 2 out of several hundreds of transformants were homologous integrants. The authors argue that the real homologous integration frequency could be higher, because the disruption of *PIM1* in

Table 1
Transformations with circular and ends-in vectors

Species	Strain/plasmid	Plasmid type/method of transformation	Target/site of the restriction cuts	Length of pTarget (kb)	Number of transformants per μg DNA	Homologous versus ectopic integration	References
A. adeninivorans	LS3, G704, G1342/ pAL-HPH1	circular/Freeze or Elpo ends-in	*Aa-rDNA*	3.3	0–3 45–176	n.s. 100%	[132]
C. albicans	hOG300/pMC1, pMK3	circular/Pp	*CaADE2*	5	n.s.	3/3	[93]
	SGY-129, A81-Pu/ pSM1825	circular/Pp	*CaADE2*	2.5	5–10	n.s.	[109]
C. glabrata	BG14/n.s.	ends-in/LiAc + ss-carDNA	*TN903 neo*	0.05 0.1 0.2 0.4	500 500–1000 5000 9000	0–8% 45–80% 90% 90%	[96]
C. oleophila	I-182/p3242	circular/LiAc ends-in	*ScHIS5*	2.1	0.3 0.5–1.5	n.s. 0/6	[100]
	I-182/pGY275	circular ends-in/LiAc ends-in/Elpo	*CoEXG1*	2.5	0 0.32 125	 6/8 n.s.	[84]
C. parapsilosis	SR23/pUC19-*CpGAL1B*	circular/LiAc + ss-carDNA ends-in	*CpGal1*	1.4	28 229	plasmids unstable plasmids stable, homologous integration	[193]
C. tropicalis	Ha900/pADE2	circular/Pp	*CtADE2*	12	10–20	5/5	[194]
	SU-2/pCU3	ends-in/Elpo + sonicated ds-carDNA	*CtURA3*	2.4	45	10/10	[131]
	M1210A3/pDUH	ends-in/Pp	*CtURA3*	1.4	0.7	5/7	[135]
H. polymorpha	A16	circular/Freeze	*HppMOX*	1.4	13–20	50%	[92]
	A16 (related to CBS4732)/pHIP1, pHIP3	circular/Freeze + ss-carDNA	*ScLEU2*	2.2	5×10^3	plasmids unstable	[88]
	/pHIP11	circular ends-in	*HpAO*	0.8	10^3 10^4	0/550 5/543	
	/pHIP111	circular ends-in	*HppAO*	2.3	10^3 10^4	0/300 9/300	
	/pHIP112	circular ends-in	*HpAMO*	2.3	10^3 10^4	n.s. 2/9	
	RB8, LR9/pHU3	circular/Freeze	*HpURA3*	n.s.	2-5	0/9	[91]
P. methanolica = *P. pinus*	2997, 2999, 3000, 2337/pUC9A2	circular/LiAc + ss-carDNA ends-in	*PmADE1*	2.0	1–10 15–150	20% n.s.	[106]
Pf. rhodozyma	CBS6938/pGB-Ph9	circular/LiAc ends-in	*Pr-rDNA*	3.0	0 5	 12/15	[85]
P. stipitis	PJH *trp*5-10 *his*3-1/pSF$_{PsADH1}$	circular/Freeze	*PsADH1*	0.8	5	4/5	[99]

		ends-in			42	13/15	
		ends in but with REMI			188	18/20	
P. pastoris	n.s.	ends-in	*PpHIS4* or *PpAOX1*	n.s.	n.s.	50–80%	[23]
S. pombe	*ura4-294*/pCG1 /pCG1-lin	circular/LiAc	*Spura4*	1.8	7	6/6	[94]
		ends-in	*Spura4*		16	5/6	
	ura4-294/pJK210ura4	ends-in/LiAc + ss-carDNA	Spura4	1.8	n.s.	54/54	[97]
	leu1-32/pJK148leu1	ends-in	*Spleu1*		500–1000	154/154	
Schw. occidentalis	RJD11	ends-in/Freeze	*SoGAM1*	2.1	50–100	70%	[195]
Yam. ohmeri = *P. ohmeri*	ATCC20209/poLEU09	circular/Elpo	*YoLEU2*	>2.3	0	–	[196]
		ends-in			50	4/4	
Yar. lipolytica	ATCC20688/pLD25	circular/LiAc + sonicated ds-carDNA	*YlLEU2*	>7.0	50	n.s.	[98]
		ends-in			>5000	9/10	
	8601-1/pINA46S	circular/Pp	Random genomic EcoRI-fragment	n.s.	n.s.	3/5	[95]
	PO1A	ends-in	*YlFKS1*	n.s.	n.s.	3/11	[173]

n.s., not specified; Freeze, freeze transformation; Pp, transformation of protoplasts; Elpo, electroporation; LiAc, lithium actate transformation; ds-carDNA, double-strand carrier DNA; ss, single-strand.

haploids could be lethal. In the homothallic *P. pastoris* both, haploids and diploids may occur in the recipient culture and the diploids could survive the integration in one of both *PIM1* copies.

In *Kluyveromyces lactis*, the homologous integration efficiency was usually found to be very low. One group, however, reported a high rate, ranging from 60% to 70% [114]. The ends-out vector used in these electroporation experiments for replacement of the *PDA1* gene was furnished with 2 kb long p:target regions. However, the efficiency of homologous integration in the same *K. lactis* strain was exceedingly locus specific [115] (Table 2). Insertion mutagenesis on the *ACS1* gene was less efficient, even though electroporation and a vector with large homologous flanks were used. Disruption of *ACS2* was even less efficient. The authors found no disruptant with a vector containing long p:target flanks among several hundred transformants. Homologous integrants could be isolated only after cells were transformed with a large gene bank fragment of a homology of several kb. Integrants needed three weeks to form larger colonies among a background of microcolonies.

Interestingly, a high rate of homologous integration in *K. lactis* was also observed if a recombinant Ti plasmid was used, that was conjugatively transferred from *Agrobacterium tumefaciens* into *K. lactis* [116]. The plasmid consisted of a *KlTRP1* locus that was disrupted by a *ScURA3* gene and flanked by T-DNA. In a control experiment, the ends-out vector without T-DNA was transformed into the same Trp-auxotrophic host strain by electroporation. Comparing the rates of homologous integration into the *TRP1* gene region of the recipient, the authors found 71% of homologous integration with the conjugative system versus 1% of homologous integration with the transformation approach. They attributed this remarkable difference to either the single-strand configuration of the T-DNA or the contribution of *Agrobacterium* gene products that had been transferred together with the DNA into the yeast cells.

Striking with these results, however, Zeeman and Steensma [115] did not observe a high efficiency of homologous integration in the *KlACS2* gene of *K. lactis* when using the *Agrobacterium* system.

In *H. polymorpha*, Lu et al. [117] applied a vector endowed with the *ScLEU2* gene as a selection marker and p:target sequences of 900 and 400 bp length. Regardless of the already mentioned ARS activity of *ScLEU2*, the percentage of homologous integration was about 25%.

In *S. pombe*, the situation seems to be controversial comparing the fate of circular or ends-in vectors (Table 1) with that of ends-out vectors (Table 2). The former was integrated mainly in the homologous target, while the latter integrated mainly ectopically. The p:target regions in the ends-out vectors used by Kaur et al. [118] had only a length of 40 bp. However, if the homologous

Table 2
Transformations with ends-out vectors

Species	Strain	Vector	Target/transformation method/length of homologous flanks (kb)	Number of transformants per μg DNA	Homologous versus ectopic integration	References
C. albicans	SGY129 A81Pu	pSM1825	*CaURA3*/Pp/1.4&2.1	110	15/18	[109]
		pUR3A	*CaURA3*/0.6&3.7	3		
	SGY-484	pJM1, pJM3	*CaHEM3*/Pp/1.3&3.1	n.s.	2/4	[110]
	CA14	*URA3*-blaster	*CaSEC14*/LiAc/1.2&1.2	n.s.	6/8	[107]
	RM1000	*URA3*-blaster	*CaARG5*/Pp/1.5&1.9	n.s.	15%, 70%	[108]
	Arg-het1	PCR-product	*CaARG5*/LiAc + ds-carDNA/ 0.060&0.060	n.s.	2/18	[111]
	RM1000 *ARG5*/*ARG5*		*CaARG5*/*LiAc*+ *ds-carDNA*/ 0.060&0.060	n.s.	4/24	
	RM1000 *arg5*/*ARG5*			n.s.	1/27 in *ARG5*, 21/27 in *arg5* (see text)	
	RM1000		*CaADE2*/0.050&0.050	n.s.	3/19	
	BWP5	PCR-product	*CaARG5*/LiAc + dscarDNA/ 0.060&0.060	n.s.	3/11	[112]
	CAI4	pDC1Gura	*CaBMH1*/LiAc/n.s.	n.s.	3/10	[197]
C. dubliniensis	UM4A, UM4B	pCdMGFP2	*CdMDR1*/Elpo/0.9&0.8	300–950	11/12	[42]
C. tropicalis	SU-2	pKD1	*CtPOX5*/Pp/1.2&2.7	70	11/11	[198]
H. polymorpha	CBS4732 8V	pSML	*HpTRP3*/LiAc/n.s.	n.s.	5/250	[199]
	D2321-51	*Sac*I-fragment of pLSF22 (with *ScLEU2*)	*HpOLE1*/Elpo/0.9&0.4	104 large among 10^4 colonies total	25/104	[117]
K. lactis	CBS2366	*uraA Kpn* I digested pMJR2120	*KlTRP1*/*LiAc* 1.6&0.8	n.s.	26/234	[200]
	PM6-7A	*Pac*I/*Kpn*1 fragment of pCYC-URA3	*KlCYC1*/ Pp/0.4&0.4	n.s.	2–6/34	[201]
	JA6	*EcoR*I fragment of the *KlGGS1* region	*KlGGS1*/Freeze/1.4&0.5	n.s.	1/100	[202]
	AWJ137	*Cla*I-fragment of pRH3	*KlLEU2*/LiAc/1.43&0.2	n.s.	1/265	[203]
	GG822	*Hpa*I fragment of the *KlCBF5* region	*KlCBF5* /Elpo/0.2&0.8	n.s.	1/22	[204]
	CBS2359	*Sca*I-*Xba*I fragmentof the *KlPDA1* region	*KlPDA1*/Elpo/2.0&1.9	n.s.	12–14/20	[114]
	CBS2359 *ura*3-59	*Kpn*I digested pRAL7211	*KlTRP1*/Elpo/0,6&1.35	n.s.	2/200	[116]
		pRAL7211	*conjugative transfer*	n.s.	142/200	
	JA6	n.s.	*KlINV1*/LiAc + ss-carDNA/ 1.2&0.65	n.s.	2/100	[205]
	MW278-20C/1	PCR product	*KlCOX14*/Elpo/500&300	n.s.	1/106	[206]
	MW179-1D			n.s.	1/36	
	D1	PCR product	*KlROM1* Freeze/ 0.9&4.6	n.s.	1/17	[207]
	CBS2359	p*Klacs1::APT1*	*KlACS1*/Elpo/2.1&1.8	n.s.	7/40	[115]
		PCR product	*KlACS2*/Elpo/1.6&0.7		0/several hundred	
		7 kb gene bank fragment with inserted selection marker	*KlACS2* /Elpo/n.s.	many microcolonies, few larger colonies after 3 weeks	2/10	

P. methanolica	2337	*PmADE1*/*ScLEU2*-combination	*PmADE1*/n.s./2.1&1.25	616 large, >6000 small colonies	49/616	[106]
P. pastoris	KM71	pYMI12a	*PpAOX2*/Pp/0.7&1.1	n.s.	0.1%	[55]
	GS190	pYMI14	*PpHIS4*/Pp/0.8&0.4	2000	1%	[123]
	GS115	*Bgl*II restriction fragment of pHIL-D1	*PpAOX1*/n.s./1.0&0.65	n.s.	1-5/20	[22]
	n.s.	n.s.	*PpAOX1*/n.s./n.s.	n.s.	10–20%	[23]
	GS115	*pim1*ΔA::*HIS4*	*PpPIM1*/Elpo/1.4&1.0	n.s.	2/several hundred	[113]
P. stipitis	PSU1	pJY102	*PsADH2*/LiAc/0.45&45	n.s.	1/17	[175]
	FPL-UC7	restricted pLU9	*PsLEU2*/*LiAc*/0.4&0.4	n.s.	1/79	[176]
	FPL-U7	pNQ26	*PsCYC1*/LiAc/0.64&0.36	n.s.	1/33	[177]
	FPL-UC7	pNQ31	*PsSTO1*/LiAc/2.7&1.7	n.s.	3/18	[120]
S. pombe	L972h$^-$ or L975h$^+$	psuc1-D4	*suc1*/LiAc/1.2&1.5	57/197 stabil	15%	[105]
			Pp	4/15 stabil	0	
		pTB7-H3	LiAc	98/492	13%	
			Pp	17/109	2%	
	JZ47	derivatives of pKT119: v1	in each case *leu1*/LiAc/1.2&1.7	n.s.	109/113	[119]
		v2	1.2&0.35		91/108	
		v3	1.2&0.2		56/96	
		v5	0.2&0.2		8/47	
	ura4-Δ18	PCR products	*sts1*, *gcs1*, *gsh2*, *hmt1*/LiAc/ 0.04&0.04	60–300	1–3%	[118]
	ura4-D18	PCR products	unknown genes 1 to 8, *pom1*, *plo1*, *spn5*, *spn6*/LiAc/ 0.08&0.08	n.s.	6–91%	[77]
	WSP20	PCR products	rec8, 10 and 11/LiAc/ 0.3&0.3	n.s.	30–70%	[78]
			0.44&0.55		20–91%	
			0.44&0.52		56–100%	
Schw. occidentalis	NGA-23	pSOcycI::TRP5	*CYC1* Freeze/n.s.	n.s.	3/23	[208]
	RJD11	*Bam*HI–*Pst*I fragment	GAM1/*Freeze*/1.0&1.0	Several hundred/30 stable	4/30	[32,195]
Y. lipolytica	PO1a	*Sal*I fragment of *YlLYS1* with an integrated *YlURA3*	*YlLYS1*/n.s./0.55&1.5	n.s.	90%	[209]
	PO1d	PCR product (PUT-cassette)	*YlACO3*/LiAc/0.8&0.7	n.s.	5-6/15	[210]
	PO1d	PCR products (p*POX*-PUT-cassettes)	YlPOX1,2,3,4,5, *length n.s.*/ LiAc/n.s.	n.s.	50%	[211]
	JM12	PCR product	*YlCRF1*/LiAc/1.5&1.4		1/10	[121]
	PO1A	n.s.	*YlFKS1*/LiAc/0.9&0.9	n.s.	0/90 (compare with Table 1)	[173]
Z. bailii	1427	PCR product	*ZBYME2*/LiAc/0.040&0.040	20	2/30	[212]
			0.4&0.4	20	6/40	

n.s., not specified; Freeze, freeze transformation; Pp, transformation of protoplasts; Elpo, electroporation; LiAc, lithium actate transformation; ds-carDNA, double-strand carrier DNA; ss, single-strand.

region was longer, then a high frequency of homologous integration could also be obtained in *S. pombe* with ends-out vectors [119]. Using flanks of more than 1 kb in length, all transformants obtained with ends-out vectors carried homologously integrated vectors. Longer flanks are, however, not in every yeast sufficient for a high percentage of homologous integration of ends-out vectors, as can be seen in *P. stipitis* [120] or *Y. lipolytica* [121] (Table 2).

An interesting technique to screen for homologous integrants in *S. pombe* was recently described by McIver et al. [122]. In their "marker switch technique" the integration sites first have to be tagged in h^+ and h^- strains with selectable markers. Then, mating of the transformants makes it possible to screen the transformants for the desired clones rather quickly.

Marker recycling opens up the possibility of serial integration mutagenesis of the same gene (in diploids) or of different genes in one and the same cell. Ends-out vectors are components of such recyclable vector systems. In *P. pastoris*, Cregg and Madden [123] used the FRT/FLP system of the 2μ plasmid of *S. cerevisiae*. In diploids, such as *C. albicans*, marker recycling systems are very useful in cases where both copies of a given gene have to be disrupted [40,112,124–126]. Such systems circumvent a possible bias in serial disruptions with different integration cassettes; a phenomenon that was already mentioned above [111].

A refined integrative transformation approach for the selection of homozygous mutants in *C. albicans* was described, incidentally, by Enloe et al. [127]. In these experiments, heterozygous transformants were produced by transformation of *C. albicans* with an ends-out vector, and homozygous mitotic segregants among them were easily selected by means of their phenotype. This approach was also used to test the essential nature of certain genes. Surprisingly, the authors isolated living transformants/recombinants, even in the case of a gene being of essential nature. They showed that the disruptants contained two mutated alleles and one wild-type allele. The additional wild-type allele had probably been produced by the triplication of the gene, either by its duplication or by the establishment of a trisomic or triploid ploidy level. Complementation of an essential function of a wild-type allele by a spontaneous duplication was also assumed to occur in *P. pastoris* [113].

3. Possible rate-limiting steps

The examples specified in Tables 1 and 2 demonstrate that the efficiency of targeted insertion mutagenesis is low in most cases. There are two main reasons for this fact. The first one is the generally low efficiency of integrative transformation. The second reason concerns the ratio between homologous and ectopic integration. If this is high, as in *S. cerevisiae* and a few other yeast species, then the transformation frequency by itself is rate limiting. That means the efficiency of the mutagenesis is similar to the transformation efficiency. On the other hand, if ectopic integration is preferred instead of homologous integration, then the efficiency of targeted insertion mutagenesis is lower than the efficiency of transformation. In every case, the yield of homologous integrants can be enhanced by an improvement of the transformation efficiency.

In the following chapters, we will summarize the factors that may be responsible for rate limitation of both integrative transformation and homologous integration.

3.1. The pathway of the vector through the cell

Homologous integration of vector DNA is the final stage of a multistep process. Before we treat molecular integration mechanisms, we should have a closer look at the events happening to the vector prior to its integration. The entrance of the vector DNA through the cell boundaries, the passage through the cytoplasm, the entrance into the nucleus and finding its target sequence all precede the establishment in the genome.

One rate-determining factor could be the amount of vector DNA available for entry into the cell. In most transformation experiments 10^7–10^8 cells are mixed with 1–10 μg of DNA, which corresponds to about 2×10^{11} up to 2×10^{12} vector molecules, assuming a vector size of 4 kb. As a result, the "vector dose" ranges between 2×10^3 and 2×10^5 molecules per cell. This means a surplus of transforming DNA in most cases. For example, Plessis and Dujon [128] reported in electroporation experiments in *S. cerevisiae* that the transformation efficiency decreased with an increase in DNA concentration. Starting with 5 or 10 ng of ends-in vector DNA per 10^8 cells, the transformation efficiency decreased significantly. Coincidently, they observed an increase of the DNA amount with multiple homologous integrations. However, the use of 500 ng of the same vector in its circular form gave no transformants. Transforming an episomal vector by the LiAc method in *S. cerevisiae*, Gietz and Schiestl [58] did not observe an increase of the transformation efficiency if the DNA amount was higher than 1 μg DNA per 10^8 cells. In *S. pombe*, Grallert et al. [105] did not find any DNA concentration-dependent difference of transformation efficiencies using either protoplasts or the LiAc method and 1–10 μg of an integrative vector.

DNA uptake from the environment depends on the competence of the cells. Natural competence for DNA uptake from the environment as it is known from bacteria [129] is not known from yeasts until now. Artificial competence, on the other hand, is reached by means of different methods, for example induction with lithium

salts, via the protoplast method, the freezing approach or by electroporation (reviewed in [58,60,61]). Details of the entry of DNA into the cells, being induced by these different transformation methods, are almost unknown [61]. However, uptake mechanisms are expected to be very different. Nevertheless, data of transformation efficiencies with integrative vectors were in many cases within the same order of magnitude (Tables 1 and 2), also if different methods and different yeast species were compared. In *H. polymorpha*, similar transformation efficiencies were reported when using the protoplast or the LiCl method [130]. However, the LiAc method resulted in a 10-fold higher efficiency than the protoplast method in *C. tropicalis* [87]. Rohrer and Picataggio [131] described similar efficiencies of the protoplast and the electroporation method in *C. tropicalis*. With protoplasts or LiAc-competent cells, Grallert et al. [105] observed similar transformation efficiencies in *S. pombe*. In *A. adeninivorans*, Rösel and Kunze [132] obtained similar transformation efficiencies with either electroporation or the freezing method. *C. oleophila* is an exception, because transformation with the electroporation method was 400-fold more effective than the LiAc method [84]. All these results are obviously influenced by methodical, species- and strain-specific peculiarities and therefore do not allow any general conclusion about the efficiency of DNA entry.

The assumption that the DNA entry into the cytoplasm is not rate limiting for targeted integration mutagenesis can also be deduced from a well-known observation: episomal vectors usually have a higher transformation efficiency, sometimes by several orders of magnitude, than the integrative transformation. However, in a given transformation system, the probability of entry into the cell should be similar for episomal and integrative vectors. Moreover, the large number of abortive transformants besides the low number of stable integrants if integrative vectors are used [133] indicates a DNA uptake comparable to that observed in experiments with episomal plasmids. Thus, the bottleneck is beyond the cell boundary.

The number of vector copies that simultaneously has entered a cell should also influence the transformation efficiency. Results of co-transformation experiments make it possible to roughly estimate the rate of multiple uptakes of vector molecules by one cell. Such experiments were performed in most cases with combinations of an episomal vector and an integrative vector. Frequencies of co-transformation were, for instance, 1–4% [72] or up to 55% [70] in *S. cerevisiae* (equivalent concentrations of both vectors were used), 30% in *C. albicans* [134] and 16% in *C. tropicalis* [135]. According to these data, simultaneous transformation of cells with more than one vector molecule occurs with relatively high frequency. A comparison of the expected and the observed transformation efficiencies is possible with data presented by Ostrander and Gorman [136]. A co-transformation rate of 10% was reported from transformations of *C. albicans* with two different integrative vectors. In the electroporation experiments, a transformation efficiency of 20 transformants per 10 μg DNA and 5×10^8 cells was determined. From this result, it follows that the rate of transformation of one cell with one vector molecule is $(20-2)/5 \times 10^8$, that is about 3.6×10^{-8}. According to this value, the rate of transformation of a cell with two vector molecules is expected to occur with a probability of 13×10^{-16}. This theoretical value is in strong contrast with the observed co-transformation frequency of $2/5 \times 10^8$ [136]. Therefore, one might assume that in many cases more than one vector molecule enters the cells during the competence phase and that the processes between cell entry and the establishment of the integrative status determine the final rate of transformation efficiency. However, it should be noted that these comparisons were made assuming that every cell of the transformed population is in a similarly competent status. A population with only a small portion of competent cells would also cause such a large difference between the expected and the observed rates of co-transformation.

Nothing is known about the mechanism by which the vector molecules arrive at their chromosomal target region after the cell entry. It is not known whether this passage is accomplished by passive diffusion or by a transport mechanism that puts the molecules actively forward to a region close to the DNA. If the molecules are moved passively and undirected by diffusion, then it would be a matter of randomness for the vector to come into a close vicinity with chromosomal DNA. Such randomness would decrease the probability of reaching a homologous chromosomal target for the vector and could therefore be rate limiting.

Establishment of an integrative vector after cell entry may be constrained by nucleolytic activities during the pathway through the cell. Using protoplast or LiAc transformation, Grallert et al. [105] observed similar transformation efficiencies in *S. pombe*. Surprisingly, the frequency of homologous integration was higher after LiAc transformation. This effect could be due to different nuclease levels in protoplasts, regenerating cells or cells prepared for the LiAc-transformation approach.

Some details are known about the intensity and kind of nucleolytic attacks during the way of the transforming DNA in *S. cerevisiae*. Leung et al. [137] compared the frequency of homologous integrants after transformation of a recipient with exogenous DNA with that of the HO-endonuclease-mediated release of the same DNA fragment from a chromosomal location. Since they found very similar frequencies, they concluded that the attack of nucleases on exogenous DNA in *S. cerevisiae* seems not to have any fundamental role.

On the other hand, short sequence recombination (SSR) which participates in the integration of ends-out vectors with short p:target sequences is apparently inhibited by nucleolytic cleavage in *S. cerevisiae* [138]. In normal cells, SSR occurs between short chromosomal repetitive DNA sequences (less than 300 bp), that are dispersed over the genome of all eukaryotes [139] and depends on the *RAD3* helicase. *RAD3* is also involved in both nucleotide excision repair (NER) and the initiation of transcription by RNA polymerase II [140,141]. In *rad3G595R* mutants, linear DNA fragments are more stable than in wild-type cells and integrations of ends-out vectors by SSR occur with higher frequencies in the *rad3G595R* background than in wild-type cells [142]. Apparently, the *RAD3* helicase prepares the ends of linear DNA pieces for nucleolytic attack.

In this context we have to discuss the enhancing effect of carrier DNA on transformation efficiencies, that was first described by Schiestl and Gietz [71] in *S. cerevisiae*. The authors proposed an improvement or activation of the DNA uptake as one explanation. One could also assume that carrier DNA could function as a ''nuclease buffer'', which would lower the likelihood of nucleolytic degradation of the vector DNA in a surplus of carrier DNA. Perhaps, however, this theory only holds for non-*Saccharomyces* yeasts, because in *S. cerevisiae* the transformation efficiency decreases at high vector DNA concentrations.

The more interesting aspect in this background is the activating effect of carrier DNA on mitotic recombination, leading, at least in *S. cerevisiae*, to an enhancement of gene disruption frequencies [71].

After the passage through the cytoplasm, the nuclear envelope and the nucleoplasm by means of unknown mechanisms, the vectors must recognize their homologous c:target before integration. Recognition of the target DNA is closely linked to the activity of the recombination machinery, initiated in many cases by double-strand breaks (DSBs). The free ends of linear insertion mutagenesis vectors are presumably involved in the processes of DSB repair [68]. Very likely, circular vectors also require DSB before they can be integrated. The next steps of integration are nucleolytic end processing and interaction of the single stranded 3′ends with the Rad52-, the Rad51- and the Rpa-proteins [143,144] of *S. cerevisiae* or their homologs in other yeasts.

One question is whether the vector finds its target by random collision with the chromosomal DNA or by an apparatus, which recognizes homologous sequences. Such ''recombinosome'' [145] would search for homologous regions between vector and chromosomal DNA, either genome wide or within a distinct section of the genome. Wilson et al. [146] observed that the frequency of homologous integrations of ends-out vectors strongly correlated with the number of target genes in a given cell. This effect was noticed if the copies were arranged as clusters as well as if they were dispersed over the whole genome. Therefore, the authors suggested that the recognition of homologous targets by the vectors was rather the result of a series of random collisions between vector and target than of the processive search of segments of chromosomal DNA.

Mitotic recombination between chromosomal loci does not seem to be connected with a genome-wide screen for homology either. In *S. cerevisiae*, homologous sequences that are dispersed over the genome recombine with different frequencies, depending on their chromosomal location [147,148]. Recombination frequencies were found to be two orders of magnitude higher if the sequences were localized on the same chromosome rather than in the case of a heterochromosomal localization [147]. Although pairing of homologous chromosomes in *S. cerevisiae* during mitosis has not yet been proven, these results show that not every chromosomal locus of the genome hits every other chromosomal locus with the same likelihood. Haber and Leung [149] did not find chromosome territoriality in connection with recombination in *S. cerevisiae*. However, they compared frequencies of single-strand annealing, a recombination mechanism that differs from the DSB repair mechanism that likely participates in the vector integration (see below).

Another fact supports the hypothesis that plasmids hit their homologous target region randomly: It is the correlation between the homologous integration efficiency and the length of the p:target region, that was observed by several authors [147,150,151]. The larger the p:target region the higher the probability that the vector hits the homologous c:target region.

3.2. Homologous integration

The integration of targeting vectors into the homologous site occurs via mitotic recombination, a process which involves products of several genes. The recombination frequency can apparently be influenced by the procedure of transformation. Higgins and Strathern [152] found that electroporation stimulated recombination in *S. cerevisiae*. Grallert et al. [105] observed a higher frequency of homologous integration after LiAc transformation than after transformation with protoplasts in *S. pombe*. As mentioned above, mitotic recombination is possibly enhanced by single-stranded carrier DNA [71].

The mechanisms of mitotic recombination have been studied so far almost exclusively in *S. cerevisiae*, but not in other yeasts. Therefore, all details that are reviewed in this chapter have been received from baker's yeast.

Mitotic recombination is closely linked with the activity of DNA double-strand break repair mechanisms and vice versa (see [68,69,153,154] for recent reviews). All integration events have in common that they depend

on genes that are members of the subdivisions of the *RAD52* epistasis group [68,138]. Homologous integration is remarkably reduced in *rad52* mutants [155,156].

Generally, mismatches between p:target and c:target reduce the frequency of homologous recombination [157–159]. The Rad51 strand transfer protein behaves in a very stringent way and tolerates only heterologies that do not exceed 9 bp [160]. Integration frequency of circular plasmids is lowered by sequence mismatches between the p:target and the c:target [151]. Disruption of the mismatch repair gene *MSH2* enhances recombination between mismatched DNA targets [158].

Integration of either circular, ends-in or ends-out vectors is very often indicated by drawing crossover symbols, as also shown in Figs. 1 and 2. These recombination events are actually found to be connected with the integration of ends-in constructs, although a number of results support the assumption that crossovers do not essentially occur during integration of ends-out vectors.

In the following part, we will compare a number of integration processes that apparently differ to some extent in their genetically determined mechanisms. These differences may be responsible for the different transformation efficiencies described by Hastings et al. [66]. The authors transformed ends-in vectors and ends-out vectors (containing p:target sequences with the same sequence and length) into the same genetic background and found a 2–3-fold higher recovery rate of ends-in experiments in comparison to ends-out experiments. Leung et al. [137] also reported a 20-fold higher efficiency of ends-in integrations in comparison with ends-out integrations. These various efficiencies may reflect differences in the integration mechanism of either ends-in or ends-out vectors.

Figs. 3–5 show integration models of different vector types. [68,69,138]. The prevailing DSB break repair model of circular or ends-in vector integration traces back already to the suppositions of Orr-Weaver et al. [64] and Szostak et al. [67] (Fig. 3). Originating from the DSB, free 3′ ends are formed by 5′–3′ exonucleolytic cleavage (resection). These free ends are recombinogenic and may invade a homologous template. They form a D-loop for subsequent replication, resulting in two Holliday structures (Fig. 3(a)). Their resolution may occur in two different ways by four endonucleolytic cuts in each case (Fig. 3(b)). Cutting and pasting results in either the release of a repaired circular plasmid molecule or in the additive integration of the plasmid (Fig. 3(c)). In brief, this type of integration is connected with replication and the formation of Holliday structures that are resolved by the recombination machinery of the cell.

Leung et al. [137] studied the integration of ends-out DNA with a length of 2–4 kb into heteroallelic chromosomal target sequences of recipients with a defect in the mismatch repair gene *PMS1*. In many cases, the transformant colonies of the *pms1*Δ cells formed two

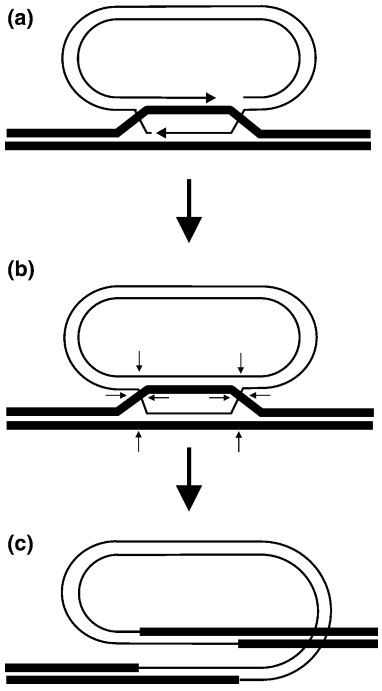

Fig. 3. Model of vector integration by the DSB repair mechanism of the cell (according to [68,69]). (a) Free 3′ ends originate by resection, invade a homologous template and form a D-loop for subsequent replication (direction indicated by arrows). (b) Their resolution may occur in two different ways by four endonucleolytic cuts (indicated by small arrows) in each case. (c) Cutting and pasting results in the additive integration of the plasmid. The chromosomal DNA is marked as thick black lines and the vector DNA is shown as thin black lines.

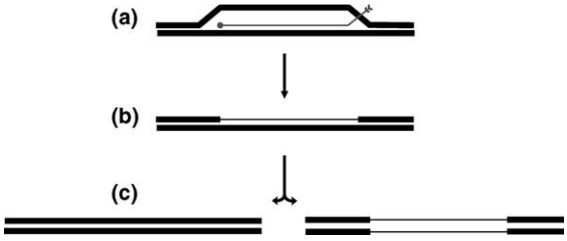

Fig. 4. Model of single-strand vector integration (according to [68,137]). The linear vector with a heteroallelic region in comparison to the target is converted into a single-stranded form. (a) The ssDNA molecule invades the double strand of the target DNA. (b) A heteroduplex structure occurs. (c) The heteroduplex region is repaired, resulting in either the target allele or the vector allele. The chromosomal DNA is marked as thick black lines. The points mark the 3′ end and the arrow tails the 5′ end of vector DNA.

sectors, one allelic with the donor and the other allelic with the recipient strain. Therefore, the authors suggested that integration had occurred after the assimilation of the vector as a linear single-strand DNA (Fig. 4(a)) and the formation of a transient heteroduplex structure (Fig. 4(b)). The heteroallelic parts of the heteroduplex were not repaired in the *pms1* cells, but they segregated during the first mitosis forming the

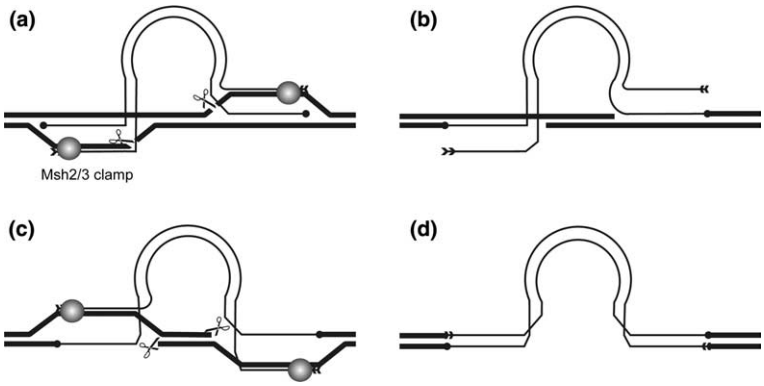

Fig. 5. Model of vector integration under the participation of the SSR mechanism (according to [138]). (a) 3′ ends displace the target strand of the same polarity. Msh2/3 bind to the structure and function as a clamp to stabilize the D loop. Nucleases as Rad50, Mre11, Xrs2 or Rad1/10 (scissors) create free 5′ ends. (b) These 5′ ends are substrates for chewing of the target DNA until that part is in register with the 5′ end of vector DNA. Both 3′ ends are ligated with the target DNA. (c) and (d) Similar processes as described in (a) and (b) lead to the complete integration of the vector. The chromosomal DNA is drawn in thick black lines. The points mark the 3′ end and the arrow tails the 5′ end of vector DNA.

two-sectored colonies. In wild-type *PSM1* cells no sectored colonies were observed because the sequence mismatches between both alleles had been repaired. These results suggest that a linear ends-out DNA fragment not necessarily integrates by two crossovers into the homologous target.

Another model of ends-out integration is based on results obtained from investigations of the SSR mechanism already mentioned above [138,139,142,161–163]. The frequency of SSR depends on the length of the substrate, noting that shorter sequences recombine remarkably less frequent than longer recombination substrates [147,150,151]. There are mechanisms that constrict SSR. They include gene products of the NER apparatus, such as Rad3p, Ssl1p and Ssl2p. Certain alleles of these genes cause enhancement of SSR. In most cases, the DNA fragments used for investigation of SSR are ends-out vectors that were constructed in the same way as the common one-step gene disruption vectors. According to these experiments, Manthey and Bailis [138] proposed the model of integration of ends-out DNA, shown in Fig. 5. In this model, integration is accompanied with cutting, chewing and pasting of DNA and seems to be independent of DNA replication, formation and resolution of Holliday structures.

Morrow et al. [164] demonstrated that for *S. cerevisiae* cells interactions between vector and chromosome can lead to much more complex consequences than simple integration. They found that a centric chromosome fragment can be formed by break-induced replication (BIR). The consequence of the associated break copy duplication in a haploid cell would be in most cases lethal. Aneuploidy would originate in diploid cells.

3.3. Illegitimate integration

In cells, mechanisms of illegitimate and homologous recombination may compete for incoming vector DNA, resulting in either homologous or ectopic integration. Illegitimate integration was investigated mainly in *S. cerevisiae*. Schiestl and Petes [101] transformed cells with *Bam*HI restriction fragments without any larger homology to chromosomal DNA of the recipient, and observed mainly integration in very short sequences with 4 bp homology to the *Bam*HI site, having a length of 6 bp. They designated this process as ''illegitimate integration'', which means that it is connected with illegitimate recombination between targets with very small or no sequence homology. Schiestl et al. [165] demonstrated that in contrast to homologous integration this type of integration does not strongly depend on most gene products of the *RAD52* epistasis group, with the exception of Rad50p. In *rad50* mutants the illegitimate integration frequency was lowered. Tsukamoto et al. [166] confirmed, with a different detection system, the influence of *RAD50*. Moreover, they found that in *rad52*, *mre11 and xrs2* mutants the frequency of illegitimate recombination was also reduced. They suggested that the physically associated proteins Rad50, Mre11 and Xrs2 [167] are part of a double-strand repair pathway that is not only involved in homologous recombination. On the other hand, the yeast Ku-protein homolog Hdf1 was found to participate in illegitimate recombination, but not in homologous recombination [166]. Meanwhile, it is well-known that these genes together with others, are part of the non-homologous end-joining (NHEJ) repair pathway of DSBs [168]. *YKU70 (=HDF1)* and *YKU80* participate in NHEJ. As *RAD50*, *MRE11* and *XRS2* the genes are also responsible for the illegitimate integration of T-DNA from the Ti-plasmid of *Agrobacterium tumefaciens* into the yeast chromosome [169,170]. Moreover, illegitimate integration obviously depends on topoisomerase I activity in *S. cerevisiae* [171]. The frequency of illegitimate recombination is remarkably enhanced by overexpression of the *TOP1* gene, but its

deletion diminishes illegitimate integration. Top1p cleaves one DNA strand mainly at the hot spot sequences [(G/C)(A/T)T] and 44% of illegitimate integrants showed integration of the non-homologous vectors into these hot spots.

Even type II topoisomerases are involved in illegitimate recombination in yeasts such as *S. pombe* [119]. In *S. cerevisiae*, Top2p inhibition by podophyllotoxin derivatives stimulates illegitimate integration [172].

4. Specific behaviour of non-*Saccharomyces* yeasts

As already mentioned above, the transformation efficiencies found in *S. cerevisiae* with circular or ends-out vectors are lower than those obtained with ends-in vectors. In each case, the efficiencies are remarkably lower than those gained with autonomously replicating plasmids. All vector types are mainly integrated in homologous regions in *S. cerevisiae*. The frequency of homologous integration increases with the length of the recombination substrate, i.e., with the length of p:target in all vector types.

Some of these characteristic features were also found in *S. pombe* or non-conventional yeast species. However, in some cases there are also distinct differences. Transformation efficiencies with ends-in vectors have been described to be very high in *C. glabrata* [96], and in one strain of *S. pombe* [97] or *Y. lipolytica* [98]. The action of carrier DNA could be responsible for the very high efficiencies of integrative transformation observed in *C. glabrata*. Moreover, with the length of p:target the frequency of transformants increased in *C. glabrata* in an exponential way. The percentage of homologous integration also increased remarkably with the length of the recombination substrate. The fact that transformants were mitotically stable excludes an episomal status of the plasmids. Therefore, it seems that *C. glabrata* possesses the most effective system for homologous integrative transformation that is known among non-*Saccharomyces* yeasts. With regard to the transformation efficiencies it could even exceed *S. cerevisiae*, although data for a direct comparison are not yet available. It would be very interesting to find out more about the characteristics of this system, especially about mitotic recombination frequencies and strength of nucleolytic activities.

In a number of yeast species, like *C. oleophila*, *C. dubliniensis*, *C. tropicalis*, *Paffia rhodozyma*, *Schwanniomyces occidentalis* and *S. pombe*, homologous integration occurs with high percentage. In *C. tropicalis*, data exist from transformations with circular, ends-in and ends-out vectors and in all cases mainly homologous integration occurs. The situation for *Y. lipolytica* is similar, although in general the percentage of homologous integration seems to be somewhat lower.

Apparently, the efficiency of homologous integration depends on the vector type used. Leon et al. [173] reported a very low efficiency of homologous integration into the *YlFKS1* gene if an ends out-vector was used. The authors described efficient homologous integration into the same locus when using an ends-in vector.

In *C. albicans*, an allele-specificity of gene targeting was observed [174]. Integration occurred with the same frequencies in two allelic loci. However, a strong preference of integration into a heteroallelic recipient was reported: either in the allele that was already disrupted or into the wild-type allele. The preference depended on the specific gene that had to be disrupted. This effect could be related to the observations made by Wilson et al. [111], as previously described.

In *S. pombe*, high frequencies of homologous integration occurred if ends-out vectors with long p:target sequences were used [78,119], whereas low homologous integration frequencies were observed with short p:targets. This could perhaps indicate a suppressing effect of SSR on recombination, as mentioned above for *S. cerevisiae*.

In *P. stipitis*, homologous integration occurs more frequently with ends-in vectors than with ends-out vectors. Cho and Jeffries [175], Lu et al. [176] and Shi et al. [120,177] observed mainly ectopic integration with ends-out vectors in experiments with four different targets and relatively long p:targets between 400 and 2700 bp. Using REMI and an ends-in vector with similar substrate length, up to 87% homologous integration was detected into the *PsADE1* gene [99].

In a further group of yeasts, homologous integration occurred with very low frequencies, generally regardless which of the vector types were used. This group includes *H. polymorpha* (although only on a small number of data from experiments with vectors without presumptive ARS sequences was available) and *P. pastoris*.

In *K. lactis*, ends-out vectors integrate with very low frequencies into homologous sites. The percentage is about 1%, even if relatively long homology stretches were used (Table 2). In this yeast, either homologous integration of this vector type may be strongly suppressed, or illegitimate recombination is preferred or the nucleolytic cleavage of incoming foreign DNA may be very high. The latter supposition is supported by the results with T-DNA of *Agrobacterium tumefaciens* that were described above. The frequency of homologous integration was very high if the vector-DNA was flanked by Ti-plasmid sequences. It was much lower in the case of transformation with the same ends-out vector without foreign flanks. The authors explained this result either by an effect of the bacterial proteins that were transferred in the yeast cells during the conjugative transfer, or by the circumstance that the transferred DNA was single-stranded. However, it could also be true that the T-DNA flanks protect the central vector

sequence in such way that it can reach the homologous target and may integrate without damage of their p:targets.

One drawback of the application of ends-in vectors is the mitotic instability if the vectors are, as in most cases, additively integrated. They can be excised, leaving behind an intact coding region at the original target site [50]. Hinnen et al. [50] described reversion of the target gene to the wild-type in *S. cerevisiae* with a rate of about 10^{-2}, but Alani et al. [79], Schiestl et al. [178] and Carls and Schiestl [179] reported a spontaneous excision rate of an additively integrated vector of about 10^{-4}. In *C. albicans*, Gorman et al. [180] determined a rate of excision of 6×10^{-6}. In *P. stipitis*, no revertants were found among 2000 *ADH1* -disruptants, corresponding to a rate of $<5 \times 10^{-3}$ [99]. Possibly, the different ranges of the data reflect differences in spontaneous homologous mitotic recombination frequencies among yeasts.

Although the instability of the constructs may be disadvantageous especially in selection procedures, integration of ends-in vectors can be a suitable tool in case where the phenotype of a disruption of a certain gene has to be studied. In such cases, only the main part of the population has to be mutated and the minor wild-type cells will be hardly noticeable. This principle is also true for targeted mutagenesis of mammalian cells, that is effectively performed using ends-in vectors [53].

In *H. polymorpha*, *K. lactis*, *P. stipitis*, *P. pastoris* or *Z. bailii*, where homologous integration of ends-out vectors occurs with low frequencies, the possibility of a more effective integration of ends-in vectors offers, combined with REMI, an alternative method to study the consequences of gene disruption.

The reasons for the low frequency of homologous integration in some yeast species are poorly understood so far. Some points that may be important here are a higher tolerance of homologs of the *Sc*Rad51 or *Sc*Msh2 proteins to DNA mismatches, a higher activity of the NHEJ pathway in comparison with the DSB repair pathway, a higher activity of the illegitimate recombination apparatus or a SSR system with a lower efficiency than in *S. cerevisiae*. All of them could represent a high risk for the cell due to enhanced genomic instabilities and less efficient DNA repair systems [181]. The preferential ectopic integration of transforming homologous DNA is obviously tolerated by some non-*Saccharomyces* yeasts, although it should be mutagenic. It is possible that these yeasts tolerate a lower stringency of their mitotic homologous recombination apparatus at the expense of a higher mutability. On the other hand, higher mutability in a moderate range would be beneficial, because it results in a higher genetic variability and thus a higher potential for genetic adaptation of a yeast population to changing environmental conditions.

In this context, it might be important to consider mutagenic effects of the carrier DNA that is added in many transformation protocols. A less stringent homologous recombination background could enable the illegitimate integration of non-homologous DNA.

5. Manipulation of the mitochondrial genome by targeted insertion

We previously described the fact that in vitro manipulated genes can be directed into the nuclear chromosomes via homologous integration as a pre-eminent tool for forward or reversed genetic analysis of nuclear gene organization, function and expression. In *S. cerevisiae*, and to date only in that yeast, similar approaches based on homologous recombination have been established for the mitochondrial genome. A prerequisite for these approaches was the development of the biolistic technique to deliver exogenous DNA into the mitochondria [182–184]. The subsequent insertion of the DNA into the target within the mitochondrial genome occurs by the high-efficient homology-dependent recombination in the mitochondria of budding yeast.

Mitochondrial (mt) transplacement in yeast was successfully used to investigate both the structural requirements for catalytic RNAs in budding yeast mitochondria such as the RNase P RNA [185] and the self-splicing of group I and group II introns [186,187] by manipulations of the desired coding sequences. Also, this technique was used to study the role of 5′ untranslated leader sequences in mitochondrial transcripts during translation control [188,189].

The exogenous DNA to be introduced into the mitochondria is usually precipitated to tungsten or gold microprojectiles [183]. In general, circular plasmids as well as linear DNA fragments can be used. However, because the frequency of mt transformation is very low, typical mt experiments involve either transformation or cotransformation of a plasmid that contains the DNA of interest, a selectable nuclear marker gene (e.g. *URA3*) and often a mitochondrial marker gene (e.g. *COXI* or *COXII* that usually complement a mt defect in trans), either on the same plasmid or on separate vectors. The nuclear marker allows initial selection for nuclear transformants. Mitochondrial transformants are typically 0.001 up to 0.05% of the nuclear transformants [190,191].

The most versatile and thus commonly used method for putting a mutant version of a mitochondrial gene or a foreign DNA flanked by homologous mtDNA sequences into the mt genome, is to first introduce the DNA of interest into cells lacking mtDNA (rho °). These primary recipients replicate, similar to naturally occurring rho$^-$ cells, the introduced DNA even if it lacks putative origins of mt replication and are therefore termed as synthetic petites. The passenger DNA is transmitted to progeny cells during vegetative growth [182]. The synthetic petites then serve as a donor in a

cross with the final recipient. During the mating, mitochondria from both parental strains fuse, forming a continuous compartment, and recombination between the introduced DNA and the mtDNA of the recipient strain lead to the desired transplacements. Consequently, cells of the resulting heteroplasmic strain carry a subpopulation of recombinant mtDNA molecules in which the DNA of interest is integrated by crossover events. Homoplasmic strains with the recombinant mt genotype can be generated by subsequent mitotic segregation.

Unlike the situation in the nucleus, recombination is very frequent and the method already works with as little as 50 bp of homologous sequences flanking the DNA to be altered; linear DNA fragments, having as little as 260 bp of homologous sequence flanking each side of a deletion mutation in a recipient, were sufficient to yield wild-type transformants at frequencies similar to those obtained with circular plasmids [192]. With regard to the efficiency of mitochondrial transformants, a wide variation was found among laboratory strains. Unfortunately, details about the genes involved in this process are yet unknown [190].

6. Future aspects

In contrast to *S. cerevisiae*, in many non-*Saccharomyces* yeasts non-homologous integration of mutagenesis vectors is preferred. The portion of homologous integrants can be influenced by several factors such as species-specific preferences of the various recombination systems in a cell, the transformation method, the vector type, the length of the vector target sequence, the vector amount per cell and by nucleolytic activities within the recipient. Until now, only few systematic studies exist on the processes that are accompanied with the efficiency of targeted insertion mutagenesis in non-*Saccharomyces* yeasts. There are several basic and practical reasons to initiate more research in this field: (i) In the first place, it is certainly of a more fundamental interest why the fate of external DNA is so different in various yeast species. The existence of virus-related Ty transposons and killer plasmids indicates a naturally occurring permeation of yeast cells by external DNA and RNA, at least in earlier times. Is the stringent homologous recombination system of *S. cerevisiae* a protection mechanism against integration of incoming DNA? Is there no intracellular protection system necessary in many recent non-*Saccharomyces* yeasts because the cell wall protects the cells sufficiently against external DNA that could be mutagenic? (ii) Second, many non-*Saccharomyces* yeasts have properties that make them interesting for basic, biotechnological and medical research. Efficient functional analysis by targeted insertion mutagenesis is a powerful tool for such investigations. (iii) Finally, even in higher eukaryotes, preferential ectopic integration occurs and may constrain applicability of targeted insertion mutagenesis in functional genetics. Molecular genetic reasons for that behaviour could be investigated by means of *S. cerevisiae* and *S. pombe* as model systems, because the genetics of both yeasts is very well developed.

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