View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

PERIODICUM BIOLOGORUM
VOL. 116, No 3, 241–247, 2014

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Hypothesis

Simultaneous plasmid integration: a unifying model of multiple plasmid integration into the yeast chromosome

PETAR T. MITRIKESKI[1,2]
ANA ŠIMATOVIĆ[1]
KRUNOSLAV BRČIĆ-KOSTIĆ[1]

[1]Laboratory for Evolutionary Genetics
Division of Molecular Biology
Ruđer Bošković Institute
Bijenička cesta 54, 10000 Zagreb, Croatia

[2]Institute for Research and Development of Sustainable Ecosystems
Ivana Lučića 5 (CTT-FSB)
HR-10000, Zagreb, Croatia

Correspondence:
Petar T. Mitrikeski
Institute for Research and Development of Sustainable Ecosystems
Ivana Lučića 5 (CTT-FSB)
HR-10000, Zagreb, Croatia
E-mail: pmitrik@irb.hr

Key words: *Saccharomyces*; Homologous recombination; Multiple plasmid integration; Genetic model

Received July 18, 2014.

Abstract

Recombination of non-replicative plasmids bearing yeast homology with the chromosome can integrate the plasmid molecule into the genome. Such process is also known to integrate more than one plasmid molecule leading to multiple, tandem plasmid integration. However, its exact molecular mechanism remains unknown. There are two alternative models to explain such integration. The first predicts single integration of a super-plasmid molecule and the second sequential integration of several independent molecules, but neither is able to comprehend all experimental data. Therefore, here is presented a theoretical model that unifies both prior models owing to the possibility that two plasmid molecules recombine with the chromosome simultaneously. This model was used as a theoretical tool in order to discriminate between existing alternatives extracting the sequential model as a better overall explanation.

INTRODUCTION

Targeted plasmid integration is a milestone of yeast genetics. Although illegitimate recombination is also possible outcome (see *1*), non-replicative plasmids bearing yeast homology transform the cell manly through homologous recombination with the chromosome (*1*, *2*). This can produce transformants that either have integrated the plasmid molecule into the genome creating at least one functional copy of the targeted locus (gene conversion followed by reciprocal exchange) or replaced the non-functional chromosomal allele with the functional one from the plasmid (gene conversion without reciprocal exchange) (*3*). However, this process is known to integrate more than one plasmid molecule into the genome (*4*). On the other side, linearizing the plasmid molecule into the homology greatly increases the targeted insertion (*5*, *6*) also frequently leading to multiple, tandem plasmid integration (MTPI) (*6*, *7*, *8*, *9*, *10*). However, the molecular mechanism of such genetic outcome of the targeted plasmid integration is not entirely understood. There have been several attempts to answer this complicated question, but despite all it still remains unsolved (*7*, *9*, *10*, *11*). Elucidating the actual mechanism of MTPI might be of fundamental interest in genetics but also offers practical advantages since multiple integration

of gene vectors is quite important in fungal systems utilized in biotechnology (see *12*).

Here we propose a theoretical model of simultaneous, multiple, tandem integration of plasmid molecules into a chromosome in order to discriminate between the sequential- (*7*) and multimer-integration pathway (*10*). Its purpose is to theoretically evaluate the probabilities of each of the existing models. This model differs from the present models due to (*i*) excluding the formation of plasmid multimers prior to recombination with the chromosome (*10*) and (*ii*) concomitantly rejecting sequential integration (*7*, *10*). Rather, it contemplates the possibility that several plasmid molecules recombine simultaneously with the same chromosomal locus due to remaining partially paranemically attached to it.

CONTEMPORARY KNOWLEDGE ON THE PROBLEM

MTPI was first reported in *S. cerevisiae* (*4*, *6*, *7*, *8*, *9*, *10*, *13*), but it is known to occur also in non-Saccharomyces yeasts (*12*, *14*, *15*, *16*, *17*) and mammals (*11*, *18*). The frequency of yeast MTPI was reported to be high (up to 50%) with double-stranded plasmids during homologous recombination (*5*) and very high (70% on average) with single stranded (ss) plasmids during illegitimate recombination (*13*). In other fungi, MTPI is usually rare in *Hansenula polymorpha* (*12*) but is known to be present in up to 16% of *Kluyveromyces lactis* transformants (*16*). On the contrary, the frequency of MTPI in mammals is very low (*11*). However, this might be dependent on the amount of used plasmid DNA since others showed decreasing frequencies with the decreased amount of DNA (*10*). The number of plasmid copies integrated into the chromosome locus varies from only two or several (*7*, *8*) to 20 copies (*9*) in *S. cerevisiae* to up to even hundred in non-Saccharomyces yeasts (see *12*). In mammals, the copy number has stabilized at only two (*11*). Additionally, it was shown that MTPI might be stimulated in specific genomic regions such as telomeres (*8*, *12*) or by specific chromosome sequences such as ARS in *S. cerevisiae* (*8*) and *Hansenula polymorpha* (*12*) or δ in *Kluyveromyces lactis* (*15*). Apart from double-strand break (DSB), the MTPI is stimulated also by psoralen (*9*) which is known to damage the hereditary material. Finally, recent experiments presented evidence that MTPI was actually increased during targeted integration with plasmids bearing terminal heterology (*19*). This suggests that multiple integration may benefit from longer duration of the recombination reaction.

There are two genetic models to explain multiple integration of plasmid molecules in tandem array into an eukaryotic chromosome during homologous recombination (Figure 1). The first one supposes that each plasmid molecule integrates by separate recombination reaction (sequential or independent integration) (Figure 1A; *7*, *10*). This model was experimentally sufficiently corroborated when it was shown that gapped plasmids all integrate as filled molecules (*7*). Thus, possibility that cytoplasmatic plasmid ligation creates super-molecule prior to recombination (that subsequently enters the chromosome) was here partially excluded. This model was also supported by experiments of illegitimate recombination in yeast where ss-plasmids were used for transformation and expectedly no evidence of cytoplasmatic ligation was detected indicating sequential multiple, tandem integration (*13*), and also by analysis of mammal recombination (*11*). Moreover, religation of even a single plasmid molecule was found to be extremely rare in yeast *S. cerevisiae* which also nicely corroborates the independent integration (*19*).

The alternative model of MTPI postulates cytoplasmatic formation of super-molecule that later integrates into the chromosome as such (non-independent integration) (Figure 1B; *10*). This possibility was experimentally supported by transforming diploid yeast strains (*10*). In those, all co-integrations occurred in only one of the two homologous chromosomes, leaving the other one intact. This strongly supports the dependant integration scenario since independent recombination events would spread on both chromosomes. Indeed, evidence of cytoplasmatic plasmid ligation prior to recombination was reported on earlier (*20*) but such results are in complete disagreement with the recent one from more elaborated experimental system (*19*). Obviously, this issue needs to be addressed further. Nevertheless, others using different experimental systems in yeast also highlighted the possibility of extra-chromosomal plasmid recombination joining two or several molecules into one that subsequently enters the genome producing MTPI in a dependant manner (*9*). Moreover, such scenario was also suggested being plausible in *Fusarium graminearum* (*17*).

There are also two other possibilities theoretically able to produce MTPI. Replicated unequally paired sister chromatids preceded by targeted insertion followed by a subsequent homologous recombination would generate tandem copies of the vector. However, such unequal sister chromatid exchange (USCE) was experimentally rejected during mammalian recombination analysis (*11*). The other possibility implies that co-integrations arise by the replication of the plasmid molecule during integration. However, when yeast is transformed by two plasmids (co-transformation) the transformants showing MTPI contain both plasmids in one array (*7*). Replication as a mechanism of MTPI would not allow such genetic outcome since there the transformants may contain only one of the co-transformed plasmids.

Taken together, this evidence clearly shows that MTPI can proceed by minimally two alternative mechanisms: (*i*) either by a single integration of a super-molecule or (*ii*) by sequential integration of several independent molecules. However, whether these are separate pathways or

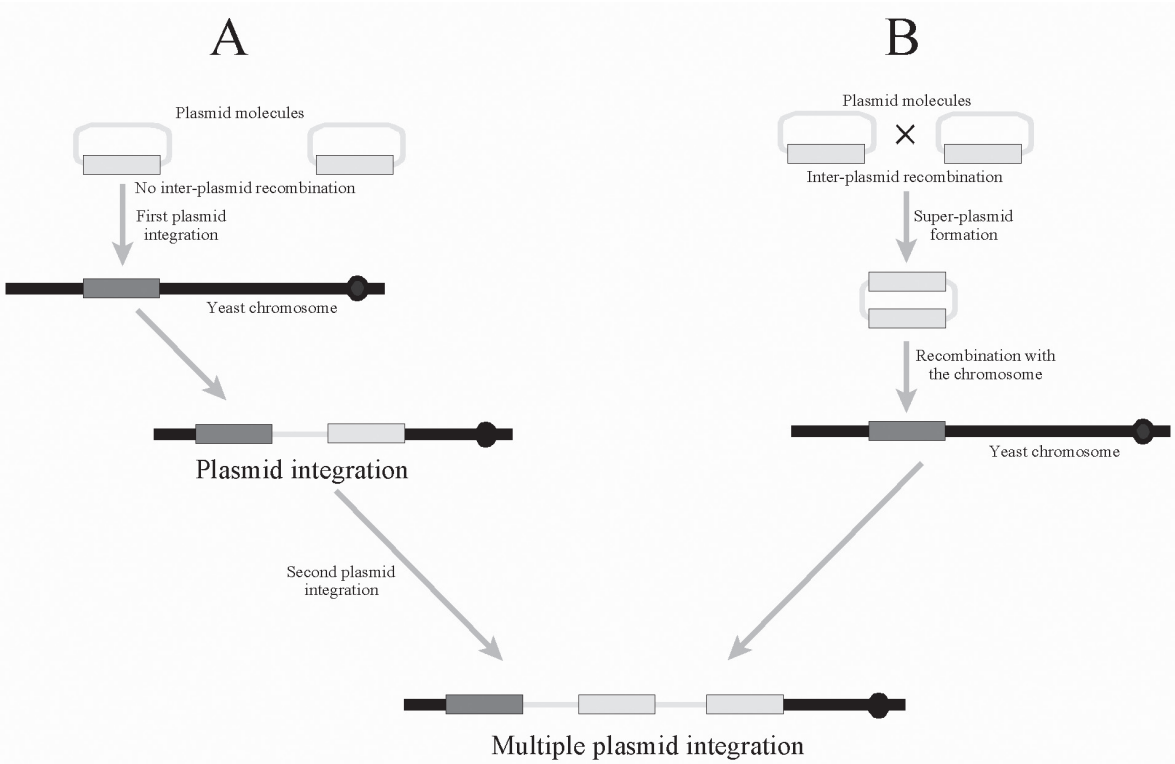

Figure 1. *Alternative models of multiple plasmid integration into the yeast chromosome (7, 10). A. Sequential or independent integration; B. Non-independent integration.*

just different mechanisms of a single cumulative pathway is not clear. If they are separate pathways what is the critical parameter to choose between them? If they are not why are there separate mechanisms?

THE MODEL

A theoretical genetic model of simultaneous, multiple, tandem integration of linearized plasmid molecules into the eukaryotic genome is presented in Figure 2. This model emphasizes (*i*) the independent homology search by each double-strand end (DSE), (*ii*) the simultaneous recombination of at least two plasmid molecules with the same chromosomal locus, and (*iii*) possible paranemic pairing (see *21*) important for later positioning of the recombining molecule(s) to the targeted homologous site. Such possibility excludes the prerogative of super-plasmid formation prior to integration, as expected by dependant model (*10*), and also renders the sequential integration, predicted by non-dependant model (*7*), unnecessary.

The general idea powering the model is the independent homology search by each DSE in all plasmid molecules dwelling the cell after transformation. Thus, if only one target homology is present in the genome, all the plasmid molecules will eventually be attracted by the same genomic locus. During this process, it is possible that two plasmid molecules establish plectonemic interaction by the same chromosomal site in a manner that different DSEs from each molecule attack separately (Figure 2A, B). Therefore two plasmid molecules are in register to simultaneously recombine with one chromosomal locus. The next step is illegitimate end-joining of the free DSEs from each molecule (Figure 2C). This will result in the formation of a super-plasmid but during recombination rather than prior to. Such super-plasmid being involved in recombination with the chromosome can be now integrated into it producing three copies of targeted genomic homology (Figure 2D).

Here is predicted that plasmid molecule(s) can be positioned on the chromosome also by the aid of paranemic pairing. However, once the homology search finishes and the plectonemic interactions are established it is difficult to leave room for paranemic pairing. On the other hand, homology that is not involved into the reaction by plectomenic pairing still remains in double-stranded form and perhaps is able to hold a paranemic assembly with the rest of the chromosome homology. Such possibility will actually make the subsequent joining of the free DSEs more plausible owing to the restrained ability of the molecule(s) to move in order to leave the reaction (Figure 2C). Thus, while the initial plectonemic positioning is necessary for establishing the recombination reaction the latter paranemic pairing may facilitate the illegitimate joining of the

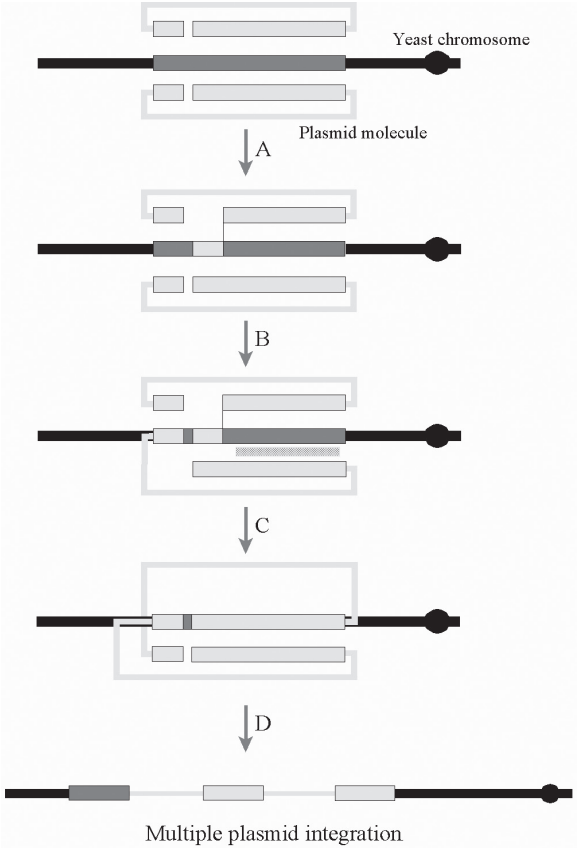

Figure 2. *Model of simultaneous, multiple integration of plasmid molecules into the yeast chromosome. A. First plasmid molecule invades the chromosomal site using only one DSE (attack with the longer end); B. Second plasmid molecule enters the reaction by invading the chromosome with its shorter end while the longer end remains paranemically attached to the chromosome; C. Branch migration involves more or even the entire invading homology of the longer end of the first plasmid molecule and subsequent illegitimate end-joining of the free DSEs from each molecule forms a super-plasmid; D. Reciprocal resolution integrates the super-plasmid into the chromosome. The dotted structure represents the paranemic pairing (see text).*

free DSEs indispensable for simultaneous integration of both molecules into the genome. If so, the suggested end-joining might be considered as homology assisted illegitimate genetic event. Similar events were reported earlier in mammals (*22*) and yeast (*19*), but however, the latter experimental system was dedicated to ends-out recombination. Nevertheless, such results directly support the idea behind the model that is presented here.

The possibility of multiple recombination during tandem plasmid integration was briefly but vaguely mentioned also by Plessis and Dujon (*10*) and elsewhere (*23*). Such a possibility of simultaneous recombination between more than two DNA molecules in a living yeast cell has indeed its experimental corroboration reported by several groups. One group demonstrated coincidental recombination events between three chromosomes initiated by a double-strand break (*24*). Evidence of a tripartite recombination involving one plasmid and two chromosome molecules was also indicated in diploid yeasts (*25*). Further, recent results indicate that a single DSB on a chromosome can search the entire genome for a homologous partner in order to complete the repair (*26*). This could lead to triparental translocations between heterologous chromosomes. Altogether, these experiments clearly demonstrate that multi-partner recombination exists in yeasts. Moreover, consequences from Ruiz *et al* (*26*) additionally suggest independent DSE homology search which nicely corroborates with the predictions of the simultaneous model.

COMPAIRING THE SIMULTANEOUS MODEL OF MULTIPLE PLASMID INTEGRATION WITH THE EXISTING MODELS

The most striking knowledge emerging from overall considerations on multiple plasmid integration arises from the fact that there is no experimental possibility to discriminate between the proposed models (when non-gapped plasmids are used; but see further chapter). Apart from circumstantial evidence (see *7*, *10*) we cannot clearly distinguish them since both reactions give the same genetic outcomes. Here, we will compare the existing models with the simultaneous model – which bears the possibility to unify them although it also produces the same genetic outcomes – in order to plausibly evaluate their individual and/or mutual weak-points and to propose which one best fits the experimental evidence.

As it was discussed before, the most serious argument supporting the independent model was presented when it was shown that gapped plasmids integrate as filled molecules (*7*). That means that all of them recombine independently with the chromosome ruling out the possibility of super-plasmid formation prior to recombination initiation. On the other hand, the possibility of non-independent integration (super-plasmid integration) was also seriously experimentally corroborated when it was shown that all integrated plasmids are located in only one chromosome copy (*10*). Moreover, the 20-fold higher reciprocal recombination between co-transformed plasmids than between plasmid-chromosome during co-transformation (*27*) and the increased frequency of co-integrations dependant on the increased amount of transforming DNA also favor the non-independent integration scenario (*10*). Therefore, while the sequential model cannot clearly explain the one-chromosome-copy plasmid location, the non-independent model is equally unable to grasp why the plasmids are integrated as filled molecules (the gap can only be filled if each plasmid molecule recombines with the chromosome separately).

The most visible explanation of one-chromosome-copy plasmid location during the sequential integration is the possibility that all plasmid molecules in the cell are eventually attracted by the recombining chromosomal site due to high protein and/or intermediates assembly (*7*, *10*). Similarly, in a diploid cell the site that first started to recombine might also attract all the plasmid molecules dwelling the cell. However, it is still difficult to imagine how the separate integration of several plasmids in more than one recombination rounds will not increase the chance of spreading the genetic events on both chromosomes. On the other side, the simultaneous model proposes *in situ* multi-plasmid recombination. Here, either all present plasmids get engaged in a multi-partner recombination reaction or most of them, being attracted by the recombining site following the integration of the first plasmid, also eventually undergo localized multi-partner recombination avoiding the alternative homologous site. This possibility would decrease the need for separate rounds of integration concomitantly reducing the possibility of multi location integration in a diploid cell. Nevertheless, both the independent and simultaneous models do not have the capacity to completely exclude such a possibility. Therefore, this dilemma is better explained by single multimer integration as predicted by the non-independent model.

On the other hand, the non-independent model cannot explain how it is possible to integrate all gapped plasmids as filled molecules since super-plasmid dependent integration excludes the possibility of filling the gap. If the initial plasmids are gapped, the multimer would be likely composed of non-filled molecules. Accordingly, the simultaneous model predicts that each molecule during multi-partner reaction interacts with the chromosome by only one DSE also ruling out the possibility of filling the gap during the recombination. Therefore, during the simultaneous recombination some of the integrated copies must be truncated contrary to the non-independent model where all of them are truncated. However, during the sequential model all recombining molecules are getting filled making separate plasmid integration the best explanation of this genetic outcome.

Altogether, this comparison clearly shows that the second dilemma (integration of filled molecules) can only be explained by the independent model contrary to the first dilemma (one-chromosome-copy plasmid location) that could better be explained by the non-independent model but, however, plausible explanation are also coming from both the sequential and the simultaneous models. Therefore, this theoretical comparison is more supportive of the independent that of non-independent model.

Is the simultaneous model plausible?

The simultaneous model presented here served as a theoretical tool to discriminate between the sequential and the multimer model of multiple plasmid integration. It appears that this model bears the power to unify the existing models owing to the possibility that several plas-

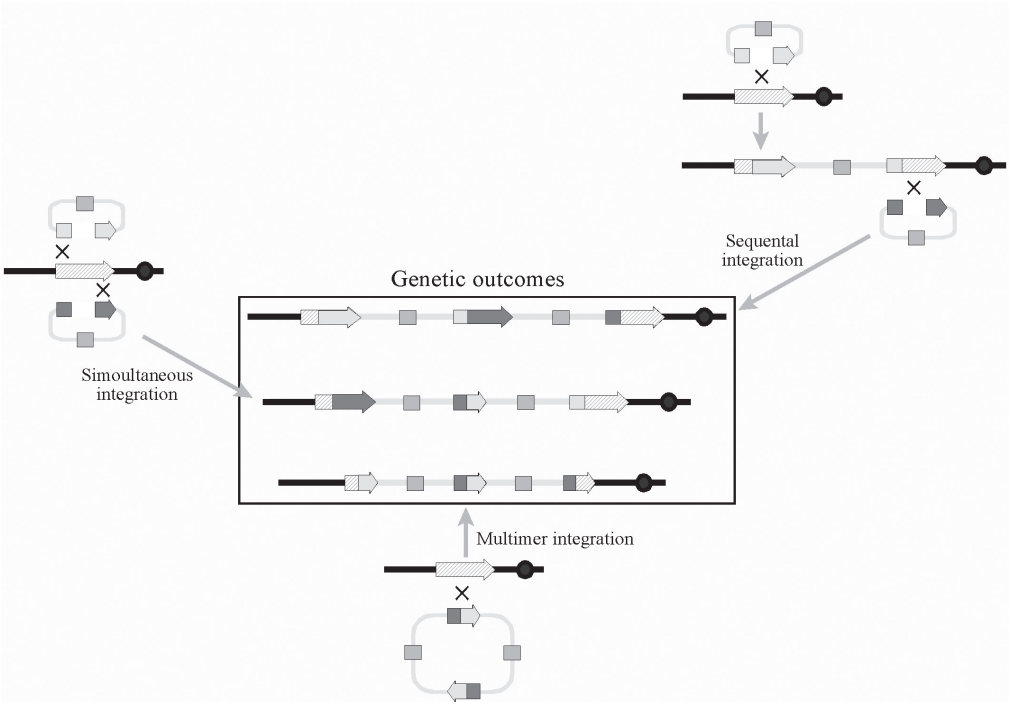

Figure 3. *Theoretical base for experimental testing of all proposed models on multiple plasmid integration. Note that not all genetic outcomes are presented due to maintaining the simplicity of the drawing.*

mid molecules simultaneously recombine with one chromosome site and thus exclude both the need for separate integration rounds and the pre-formed multimer integration. However, while the simultaneous model offers a plausible explanation for one-site integration it is also unable to clearly explain the experimental finding showing that all gapped plasmids are integrated as filled molecules. This was earlier evaluated by analyzing 14 transformants showing multiple plasmid integration (*7*). However, this number seems rather small for a definitive conclusion. While it might be that the sequential model is predominant it still does not mean it is exclusive to other models including the simultaneous model. Indeed, the simultaneous model fairly overrides the prerequisite of multimer integration and offers a possibility that not all copies of gapped plasmids integrate as non-filled molecules (as happens with the non-independent model) leaving the foundation for experimental evaluation of all proposed models.

We believe that there is no reasonable doubt that would render the simultaneous model impossible. However, unless tested experimentally we cannot clearly tell. One possibility to discriminate between all the proposed models is to use only gapped plasmids for transformation while concomitantly searching for a tandem multiple plasmid integration bearing some truncated copies of the integrated molecules (Figure 3). All truncated copies would indicate multimer integration, all non-truncated sequential integration and partial truncation would be supportive of the simultaneous model.

CONCLUSION

Targeted plasmid integration frequently proceeds by integrating more than one molecule into the genome. This could be interpreted either by dependant integration where a pre-formed super-plasmid molecule integrates into the genome or by sequential integration of several independent molecules. However, neither of these models is able to comprise all the experimental data. Therefore, in order to discriminate between the existing models here is presented a theoretical model based on the possibility that two plasmid molecules recombine with the targeted chromosome simultaneously excluding both the super-plasmid formation and the sequential integration. The theoretical comparison extracted the sequential model as a better overall explanation. However, it cannot be clearly shown that this is the only pathway of multiple plasmid integration rendering the alternative models (including the simultaneous model) also plausible. Finally, the theoretical comparison revealed a base for experimental testing of all the existing models provided that the transformation is preceded by gapped plasmids.

Acknowledgements: *We wish to thank to M.Sc. Juraj Bergman (Ruđer Bošković Institute) for the critical reading of the manuscript and improving the English.*

REFERENCES

1. ZGAGA Z, GJURAČIĆ K, SVETEC I K, MITRIKESKI P T, GREGORIĆ S 2001 Plasmid integration in yeast: conceptions and misconceptions. *In:* Kniewald Z *(ed)* Current Studies of Biotechnology – Vol. II. Environment. Croatian Society of Biotechnology, Zagreb, p 135
2. HINNEN A, HICKS J, FINK G 1978 Transformation of yeast. *Proc Natl Acad Sci USA 75:* 1929-1933
3. SZOSTAK J W, ORR-WEAVER T L, ROTHSTEIN R J, STAHL FW 1983 The double-strand-break repair model for recombination. *Cell 33:* 25-35
4. SZOSTAK J W, WU R 1979 Insertion of a genetic marker into the ribosomal DNA of yeast. *Plasmid 2:* 536-554
5. ORR-WEAVER T L, SZOSTAK J W, ROTHSTEIN R J 1981 Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci USA 78:* 6354-6358
6. ORR-WEAVER T L, SZOSTAK J W 1983 Yeast recombination: the association between double-strand gap repair and crossing-over. *Proc Natl Acad Sci USA 80:* 4417-4421
7. ORR-WEAVER T L, SZOSTAK J W 1983a Multiple, tandem plasmid integration in *Saccharomyces cerevisiae*. *Mol Cell Biol 3:* 747-749
8. HOHMANN S 1987 A region in the yeast genome which favours multiple integration of DNA via homologous recombination. *Curr Genet 12:* 519-526
9. SAFFRAN W A, SMITH E D, CHAN S K 1991 Induction of multiple plasmid recombination in *Saccharomyces cerevisiae* by psoralen reaction and double strand breaks *Nucleic Acids Res 19:* 5681-5687
10. PLESSIS A, DUJON B 1993 Multiple tandem integrations of transforming DNA sequences in yeast chromosomes suggest a mechanism for integrative transformation by homologous recombination. *Gene 134:* 41-50
11. NG P, BAKER M D 1999 The molecular basis of multiple vector insertion by gene targeting in mammalian cells. *Genetics 151:* 1143-1155
12. SOHN J H, CHOI E S, KIM C H, AGAPHONOV M O, TER-AVANESYAN M D, RHEE J S, RHEE S K 1996 A novel autonomously replicating sequence (ARS) for multiple integration in the yeast *Hansenula polymorpha* DL-1. *J Bacteriol 178:* 4420-4428
13. GJURAČIĆ K, ZGAGA Z 1996 Illegitimate integration of single-stranded DNA in *Saccharomyces cerevisiae*. *Mol Gen Genet 253:* 173-181
14. SOHN J H, CHOI E S, KANG H A, RHEE J S, AGAPHONOV M O, TER-AVANESYAN M D, RHEE S K 1999 A dominant selection system designed for copy-number-controlled gene integration in *Hansenula polymorpha* DL-1. *Appl Microbiol Biotechnol 51:* 800-807
15. WANG Y C, CHUANG L L, LEE F W, DA SILVA N A 2003 Sequential cloned gene integration in the yeast *Kluyveromyces lactis*. *Appl Microbiol Biotechnol 62:* 523-527
16. READ J D, COLUSSI P A, GANATRA M B, TARON C H 2007 Acetamide selection of *Kluyveromyces lactis* cells transformed with an integrative vector leads to high-frequency formation of multicopy strains. *Appl Environ Microbiol 73:* 5088-5096
17. WATSON R J, BURCHAT S, BOSLEY J 2008 A model for integration of DNA into the genome during transformation of *Fusarium graminearum*. *Fungal Genet Biol 45:* 1348-1363
18. ANDERSON R A, KRAKAUER T, CAMERINI-OTERO R D 1982 DNA-mediated gene transfer: recombination between co-transferred DNA sequences and recovery of recombinants in a plasmid. *Proc Natl Acad Sci USA 79:* 2748-2752

19. SVETEC I K, ŠTAFA A, ZGAGA Z 2007 Genetic side effects accompanying gene targeting in yeast: the influence of short heterologous termini. *Yeast 24:* 637-652

20. SUZUKI K, IMAI Y, YAMASHITA I, FUKUI S 1983 *In vivo* ligation of linear DNA molecules to circular forms in the yeast *Saccharomyces cerevisiae*. *J Bacteriol 155:* 747-754

21. YAGIL G 1991 Paranemic structures of DNA and their role in DNA unwinding. *Crit Rev Biochem Mol Biol 26:* 475-559

22. SAKAGAMI K, TOKINAGA Y, YOSHIKURA H, KOBAYASHI I 1994 Homology-associated nonhomologous recombination in mammalian gene targeting. *Proc Natl Acad Sci USA 91:* 8527-8531

23. MITRIKESKI P T, GJURAČIĆ K, KOREN P, LISNIĆ B, MIKLENIĆ M, ŠTAFA A, SVETEC I K 2012 The pioneer of yeast genetics in Croatia: Zoran Zgaga's contribution to make national research acknowledged worldwide. *Period biol 114:* 1-14

24. RAY A, MACHIN N, STAHL F W 1989 A DNA double chain break stimulates triparental recombination in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA 86:* 6225-6229

25. SILBERMAN R, KUPIEC M 1994 Plasmid-mediated induction of recombination in yeast. *Genetics 137:* 41-48

26. RUIZ J F, GÓMEZ-GONZÁLEZ B, AGUILERA A 2009 Chromosomal translocations caused by either pol32-dependent or pol32-independent triparental break-induced replication. *Mol Cell Biol 29:* 5441-5454

27. ZGAGA Z, ALAČEVIĆ M 1991 Recombination between replicative and integrative plasmid in the yeast *Saccharomyces cerevisiae*. *Prehrambeno-tehnol biotehnol rev 29:* 19-23