Journal of Cell and Molecular Research (2012-2013) 4 (2), 56-61

Deletion of *RAD52* in *Saccharomyces cerevisiae* severely decreases frequencies of *Agrobacterium* genetic transformation mediated by either an integrative or a replicating binary vector

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Received 23 October 2012 Accepted 13 February 2013

Abstract

Agrobacterium tumefaciens is capable to transfer genes across kingdoms. It can genetically transform not only plant cells, but also many other bacterial, algal, fungal, animal and human cells. This depends on the interactions among a variety of both *Agrobacterium* and host genes. Inside the host cell, *RAD52* which is involved in DNA repair is a key gene determining integration of T-DNA by homologous recombination. Here, using *Saccharomyces cerevisiae* haploid strains BY4741 and BY4742, a *rad52* diploid deletion strain was constructed in yeast BY4743 background. This model organism was employed to show that *RAD52* deletion severely decreases frequencies of *Agrobacterium* genetic transformation mediated by either an integrative T-DNA or a circular non-integrative T-DNA. Indeed, the frequencies of such *Agrobacterium*-mediated transformation (AMT) decreased by *ca*. 25-fold, compared to wild type BY4743. Hence, host *RAD52* deletion might affect AMT by a mechanism which differs from its only involvement in DNA repair in yeast.

***Keywords*:** RAD52, AMT, Agrobacterium, Saccharomyces

Introduction

Agrobacterium tumefaciens is naturally a soilborne phytopathogen which genetically transforms plants and causes crown gall disease in numerous plants (De Cleene and De Ley, 1976). In laboratory, it is capable to transform cells from many non-plant organisms including yeast, *Saccharomyces cerevisiae* (Soltani et al., 2008). This ability of *Agrobacterium* is based on a large tumor inducing (Ti) plasmid, which contains a set of virulence (*vir*) genes that can mobilize a segment of the Ti-plasmid, i.e. the T-DNA. This T-DNA, in a single stranded form (T-strand), is transported to the host cell where it can integrate into the host genome. Meanwhile, *Agrobacterium* also transfers a number of its virulence proteins to the host cell through its type IV secretion system (Vergunst et al., 2000, Schrammeijer et al., 2003). Delivered virulence proteins protect the T-strand from host nucleases, target it to the nucleus and possibly cooperate with host proteins to integrate it into the host genome (Tzfira et al., 2004).

Over the last decade, the yeast *S. cerevisiae* has become an excellent model host to study the host factors involved in *Agrobacterium*-mediated transformation (AMT). One of the key groups of identified host genes which affect AMT of yeast is *RAD52* epistasis group (Soltani, 2009). AMT of *S. cerevisiae* can result in random insertion of the T-DNA into the yeast genome by non-homologous end joining (Bundock and Hooykaas, 1996). However, when DNA sequences homologous to those of *S. cerevisiae* genome are present, the DNA fragment will mostly integrate into the genome by homologous recombination. When T-DNA contains a *S. cerevisiae* replicator such as an autonomously replicating sequence (ARS) or the replicator of the 2µ plasmid, the T-DNA will be maintained in the yeast cell as a replicative plasmid (Bundock et al., 1995; Piers et al., 1996), after circularization of the T-DNA. In *S. cerevisiae*, the integration of T-DNA by homologous recombination is very efficient. By using the yeast *S. cerevisiae* as a model, it was found that the proteins mediating T-DNA integration are the proteins involved in double strand break (DSB) repair of the genomic DNA (van Attikum et al., 2001). Rad52 is essential for T-DNA integration at double strand breaks by homologous recombination (van Attikum and Hooykaas, 2003). However, Yku70 is essential for T-DNA integration at double strand breaks by non-homologous end joining (van Attikum, 2003). Thus, in *S. cerevisiae* the Rad52 and Yku70 proteins play critical roles in determining whether

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the T-DNA is integrated via homologous recombination or via nonhomologous end joining (van Attikum, 2003). Hence, it is speculated that *RAD52* deletion in host cell might affect T-DNA integration by influencing DNA repair system. However, the effect of *RAD52* deletion on AMT is not investigated by replicating plasmids.

Here, the effect of host *RAD52* deletion on AMT frequencies is assayed by either a homologous integrative or a replicating non-integrative binary vector. To this end, yeast *rad52* diploid deletion mutants were constructed in *S. cerevisiae* BY4743 background. Then, AMT frequencies were assayed in *rad52* deletion mutants of *S. cerevisiae*, using both a homologous integrative and a replicating binary vectors.

Materials and Methods

Bacterial strains, plasmids and media

Two derivatives of *A. tumefaciens* strain LBA1100, containing either pRAL7100 (integrative T-DNA) or pRAL7101 (replicating T-DNA) binary vectors (Bundock et al., 1995), were used. The T-DNA of pRAL7100 contains the *URA3* gene flanked by the yeast *PDA1* sequences that allows integration into the yeast *PDA1* locus via homologous recombination. The T-DNA of pRAL7101 contains the *URA3* gene and the yeast 2µ origin of replication. Following AMT the T-DNA part of this plasmid will be maintained in yeast nucleus as an extrachromosomal replicating plasmid after circularization. *Agrobacterium* was grown and maintained as described by Hooykaas et al. (2006). *Escherichia coli* XL1-Blue was used for plasmid amplification.

Yeast strains and media

Saccharomyces cerevisiae haploid strains BY4741 (*MAT*a *his3Δ leu2Δ met15Δ ura3Δ*) and BY4742 (*MATα his3Δ leu2Δ lys2Δ ura3Δ*), obtained from Invitrogen (Groningen, the Netherlands), were used as the wild type strains to construct yeast diploid strain BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ/leu2Δ lys2Δ/LYS2 MET15/met15Δ ura3Δ/ura3Δ*), and to construct *rad52* diploid deletion strain in BY4743 background. Yeast BY strains were grown in liquid YPD medium at 30°C and *rad52* deletion mutant strain was grown in liquid YPD medium containing G418 (150 µg/ml) at 30ºC.

Construction of rad52 diploid deletion strain

To construct *rad52* diploid deletion strains in yeast BY4743 background, primers P3 and P4 (table 1) were designed to amplify *KANMX* cassette (1.6 kb), flanked by *loxP*, from pUG6 plasmid (Güldener et al., 1996) and 45 bp sequences upstream and downstream of *RAD52* gene by PCR. The amplified fragment was gel purified, and was transferred to yeast haploid strains BY4741 and BY4742 by Lithium Acetate transformation method (Gietz et al., 1995) and the deletion strains were selected on YPD medium containing G418 (150 µg/ml).The integration at *RAD52* locus was confirmed by colony PCR using four primers: P5 and P6 (635 bp), and P7 and P8 (560 bp) (table 1) to check upstream and downstream of integrated fragment. Then, the *rad52* haploid deletion strains were mated (Stansfield and Stark, 2007) to obtain a homozygous diploid deletion strain in BY4743 background, and were selected on MY medium (Zonneveld, 1986) supplemented with histidine (20 µg/ml), leucine (30 µg/ml)) and uracil (20 µg/ml). Construction of diploid deletion strains were confirmed by a duplex colony PCR using the universal primers of MAT locus (Table 1) (Huxley et al., 1990).

Chemical and Agrobacterium-mediated transformation of yeast strains

The lithium acetate transformation protocol as described by Gietz et al. (1995) was used to transfer the amplified fragments to yeast BY4741 and BY4742 strains. The original *Agrobacterium*-mediated transformation (AMT) protocol (Bundock et al., 1995) was used for transformation of yeast mutants. Yeast wild type strain BY4743 was used as a control.

Results

Construction of homozygous rad52 diploid deletion mutants

To construct *rad*52 diploid deletion in yeast BY4743 diploid background, the *KANMX* cassette from pUG6 was amplified using primers with 45 bp homologies to upstream and downstream regions of yeast *RAD*52 gene, i.e. P3 and P4. The amplified fragment was evaluated by gel electrophoresis, and subsequently was transferred to yeast haploid strains of BY4741 and BY4742. Colony PCR by primer combinations of P5 and P6, and P7 and P8 amplified the expected fragments, 635 bp and 560 bp respectively, in several yeast *rad*52 deletion mutants of BY4741 and BY4742 strains (figure 1). Upon this, *rad*52 haploid deletion strains of BY4741 and BY4742 background were mated to create BY4743 diploid strains. Then, yeast diploid strains were selected on MY medium supplemented

with histidine, leucine and uracil. Colony duplex PCR by *MAT* locus primers i.e. P9, P10 and P11, amplified the expected fragments of 492 bp and 369 bp in novel diploid deletion mutants, confirming their homozygosis (figure 2). This led to the construction of *rad*52 homozygous diploid deletion mutants in yeast BY4743 background.

Table 1. Primers designed and used in this study.

Oligo	Sequence (5'-3')
Universal primers for pUG6-KanMx cassette	
P1	CCagctgaagcttcgtacgc
P2	GCATAGGCCACTAGTGGATCTG
45 bp sequences from *RAD52*	
Upstream	TTGCCAAGAACTGCTGAAGGTTCTGGTGGCTTTGGTGTGTTGTTG
Downstream	AACGCTTCCTGGCCGAAACAAATAAAAAATTTGCATCATTATTTA
Primers to amplify *KANMX*-45bp *RAD52*	
P3	TTGCCAAGAACTGCTGAAGGTTCTGGTGGCTTTGGTGTGTTGTTG CCagctgaagcttcgtacgc
P4	TAAATAATGATGCAAATTTTTTATTTGTTTCGGCCAGGAAGCGTT GCATAGGCCACTAGTGGATCTG
Primers to check for *rad52* deletions	
P5 (*RAD52*)	AATGCAAACAAGGAGGTTGC
P6 (*KANMX*)	TCAGAAACAACTCTGGCG
P7 (*RAD52*)	CGACACATGGAGGAAAGAAAA
P8 (*KANMX*)	CTTCATTACAGAAACGGCT
Universal primers of *MAT* locus	
P9	AGTCACATCAAGATCTTTATGG
P10 (*MAT*α)	GCACGGAATATGGGACTACTTCG
P11 (*MAT*a)	ACTCCACTTCAAGTAAGAGTTTG

AMT of yeast rad52 homozygous diploid deletion mutants

Using *S. cerevisiae rad*52 homozygous diploid deletion mutant as the host, AMT was performed by either pRAL7100 (containing a homologous integrative T-DNA) or pRAL7101 (containing a replicating circular T-DNA) binary vectors. Data from two experiments (with two repeats in each one for *rad*52 deletion) are represented in Table 2 and Table 3. Analyses of AMT frequencies and relative frequencies in *rad*52 diploid deletion strains in comparison with wild type strain BY4743, as control, are represented in Table 4. As seen, relative frequencies of AMT in *rad*52 deletants are decreased to *ca*. 4% of the wild type strain, both with integrative and replicating binary vectors.

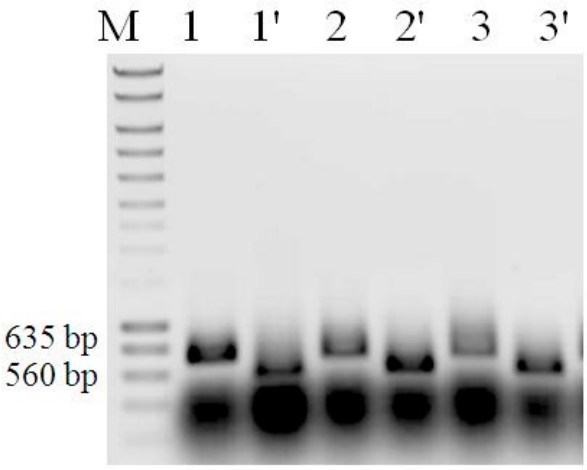

Figure 1. *RAD*52 substitution by *KANMX* cassette in deletion mutants (No.1-5) of yeast haploid strains of BY4742 (No. 1 and 2) and of BY4741 (No. 3,). Bands in lanes 1, 2, 3 obtained from primer combinations P5 and P6 (635 bp), and in lanes 1', 2', 3' obtained from primers combinations P7 and P8 (560 bp). M: DNA molecular ladder of 10 kb.

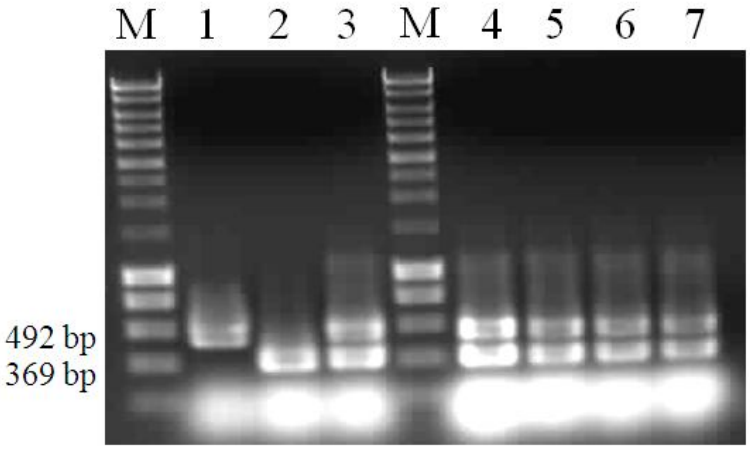

Figure 2. Presence of both *MAT*a and *MAT*α loci in yeast *rad*52 diploid deletion mutants constructed by mating haploid strains of BY4742 and BY4741. Lane 1: *MAT*a in haploid strain BY4741 (492 bp); lane 2: *MAT*α in haploid strain BY4742 (369 bp); lane 3: *MAT*a and *MAT*α in diploid strain BY4743; lanes 4, 5, 6, 7: *MAT*a and *MAT*α in *rad*52 diploid deletion strains in yeast BY4743 background. M: DNA molecular ladder of 10 kb.

Table 2. AMT of yeast wild type strain BY4743 and its *rad*52 deletion diploid strain by using a homologous integrative T-DNA.

pRAL7100 (integrative T-DNA)	Experiment 1			Experiment 2		
	Number of transformants	Output × 10^{-5}	Frequency	Number of transformants	Output × 10^{-5}	Frequency
Wt BY4743	143	385	0.371	180	228	0.789
rad*52*Δ	Repeat1: 6	102	0.059	4	162	0.025
	Repeat2: 1	97	0.010	2	203	0.009

Frequencies are depicted as the number of uracil prototrophic colonies divided by the output number of yeast cells. Transformations were performed using the standard transformation protocol (Bundock et al., 1995).

Table 3. AMT of yeast wild type strain BY4743 and its *rad*52 deletion diploid strain by using a replicating non-integrative T-DNA.

pRAL7101 (replicating T-DNA)	Experiment 1			Experiment 2		
	Number of transformants	Output × 10^{-5}	Frequency	Number of transformants	Output × 10^{-5}	Frequency
Wt BY4743	215	471	0.456	154	339	0.454
rad*52*Δ	Repeat1: 3	155	0.019	2	125	0.016
	Repeat2: 2	164	0.012	3	147	0.020

Frequencies are depicted as the number of uracil prototrophic colonies divided by the output number of yeast cells. Transformations were performed using the standard transformation protocol (Bundock et al., 1995).

Table 4. Analyses of frequencies and relative frequencies of AMT in yeast *rad*52 diploid deletion strain, performed either by an integrative or a replicating T-DNA.

	pRAL7100 (integrative T-DNA)			pRAL7101 (replicating T-DNA)		
	Average of AMT frequencies	Relative frequency	Relative frequency (%)	Average of AMT frequencies	Relative frequency	Relative frequency (%)
Wt BY4743	0.580	1.001	100	0.455376	1.001	100
	Repeat1: 0.042	0.072	7	0.017677	0.039	4
rad*52*Δ	Repeat2: 0.010	0.017	1	0.016302	0.036	4

Discussion

Agrobacterium-mediated transformation is a method of high interest for genetic transformation of both plants and fungi. In addition to agrobacterial proteins involved in AMT, it is evident from previous experiments that host proteins highly influence the fate of introduced transgene, T-DNA, thereby affecting the AMT frequencies (Citovsky et al., 2006). A group of such proteins, identified in model eukaryote *S. cerevisiae*, are *RAD*52 epistasis group of proteins (Soltani, 2009), which are initially involved in regulation of DNA double-strand breaks (DSBs) repair by homologous recombination (HR). Regulation of DSBs in the yeast *S. cerevisiae* involves the recruitment of Rad52, a central recombination protein, to sites of DNA breaks. The Rad52 protein plays a role in strand exchange and the annealing of single strand DNA, especially upon entry into S phase (Barlow and Rothstein, 2010). Thus, proper functioning of Rad52 affects double strand break repair by initiating and/or directing of many aspects of HR. It is shown that *rad*52 deletion affects the T-DNA integration by HR (van Attikum and Hooykaas, 2003). This has led to the speculation that *RAD*52 might affect AMT by influencing DNA repair by HR.

T-DNA containing a *S. cerevisiae* replicator, like ARS or 2µ replicators, doesn't integrate into the host genome. Instead, after circularization, it will be maintained in the yeast cell as a replicating plasmid (Bundock et al., 1995; Piers et al., 1996). Here, both integrative and replicating binary vectors were assayed for AMT of yeast *rad*52 deletion strains. As it is shown in Table 4, the AMT frequencies mediated by HR-integrative binary vector pRAL7100 were decreased by *ca.* 25 fold, compared to the wild type BY4743. This is in agreement with previous results reported by van Attikum and Hooykaas (2003). However, it is further found that AMT frequencies obtained by the non-integrative binary vector pRAL7101, which will be remained as a circular replicating plasmid in the yeast cell, were also decreased by *ca.* 25 fold, compared to the wild type BY4743. This raises the question about the real effect of host *rad*52 deletion on the T-DNA fate inside the cell. To address this, *RAD*52 deletion may affect the replication of introduced T-DNA, in addition to its affection on host cell integrity. In support of the first hypothesis it is shown that the Rad52 is positively regulated upon entry into S phase, but repressed during the intra-S phase checkpoint (Barlow and Rothstein, 2010). Although HR is inhibited at stalled replication forks, for cell survival in the event of fork collapse, HR is necessary during S phase (Lisby et al., 2001). This implicates the function of *RAD*52 for the replication fork restart and thus proper DNA replication. Such functions might support the proper replication of introduced replicating T-DNA as well. Hence, in the absence of *RAD*52, replication of T-DNA may be suppressed and subsequently the AMT frequencies are reduced. On the other hand, *rad*52 deletion may have complicated effects on the cell integrity which are not fully explored yet, affecting the introduction of T-DNA into the cell and/or the expression of its genes.

Acknowledgment

This research was financed by the Grant No.800363 of the MSRT of Iran to J. Soltani. The author (J.S.) wishes to dedicate this work to the memorial of his beloved mother, Robabeh Mawlaei.

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