Increased Agrobacterium-mediated transformation of Saccharomyces cerevisiae after deletion of the yeast ADA2 gene

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ORIGINAL ARTICLE

Increased *Agrobacterium*-mediated transformation of *Saccharomyces cerevisiae* after deletion of the yeast *ADA2* gene

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Significance and Impact of the Study: In this study, we have shown that deletion of *ADA2*, encoding a component of the ADA and SAGA transcriptional adaptor/histone acetyltransferase complexes, from the yeast *Saccharomyces cerevisiae* resulted in an increased efficiency of *Agrobacterium*-mediated transformation. This increased efficiency occurred irrespective of whether T-DNA integrates by homologous or non-homologous recombination. The effect on T-DNA integration by non-homologous recombination is of special importance as this process is very inefficient in *S. cerevisiae*. This result may open ways to improve transformation protocols for fungi and yeasts that are difficult to transform.

Keywords
biotechnology, fungi, rhizobia, transformation, yeasts.

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Abstract

Agrobacterium tumefaciens is the causative agent of crown gall disease and is widely used as a vector to create transgenic plants. Under laboratory conditions, the yeast *Saccharomyces cerevisiae* and other yeasts and fungi can also be transformed, and *Agrobacterium*-mediated transformation (AMT) is now considered the method of choice for genetic transformation of many fungi. Unlike plants, in *S. cerevisiae*, T-DNA is integrated preferentially by homologous recombination and integration by non-homologous recombination is very inefficient. Here we report that upon deletion of *ADA2*, encoding a component of the ADA and SAGA transcriptional adaptor/histone acetyltransferase complexes, the efficiency of AMT significantly increased regardless of whether integration of T-DNA was mediated by homologous or non-homologous recombination. This correlates with an increase in double-strand DNA breaks, the putative entry sites for T-DNA, in the genome of the *ada2*Δ deletion mutant, as visualized by the number of Rad52-GFP foci. Our observations may be useful to enhance the transformation of species that are difficult to transform.

Introduction

The soil pathogen *Agrobacterium tumefaciens* is renowned for its ability to transform a broad range of plant species (for review, see Nester *et al.* 1984; Tzfira and Citovsky 2006; Păcurar *et al.* 2011; Christie and Gordon 2014; Gelvin 2017). Under laboratory conditions, *Agrobacterium* can also transform the yeast *Saccharomyces cerevisiae* and many fungi (Bundock *et al.* 1995; Piers *et al.* 1996; de Groot *et al.* 1998; Bundock *et al.* 1999). This unique ability of *Agrobacterium* has made *Agrobacterium*-mediated transformation (AMT) not only essential for plant biology research, but also of increased importance for fungal research (for review, see Hooykaas *et al.* 2018).

The DNA segment introduced into host cells by *Agrobacterium*, T-DNA, is derived from its tumour-inducing plasmid (Ti-plasmid). The T-DNA is transferred in a single-stranded form, the T-strand, which carries the VirD2 protein covalently linked at its 5′-end. The VirD2 protein contains a nuclear localization sequence which is

necessary for translocation of the T-strand into the nucleus of the host cell (Rossi *et al.* 1993). In the host cell nucleus, the T-strand is converted into a double-stranded T-DNA. Such T-DNA molecules can then circularize (Bundock *et al.* 1995) or form more complex extrachromosomal structures (Singer *et al.* 2012; Rolloos *et al.* 2014). They can be maintained if possessing a replicator (Bundock *et al.* 1995). More commonly, stable maintenance of T-DNA in the host cell is achieved after integration into the host genome. Host factors mediate T-DNA integration and this explains why T-DNA is integrated preferably by homologous recombination in yeast and by non-homologous recombination in plant cells (Offringa *et al.* 1990; Bundock *et al.* 1995).

Which host factors play a role in AMT is still far from clear. Importins from the host cell mediate import of the T-strand into the nucleus (Ballas and Citovsky 1997; Bhattacharjee *et al.* 2008). T-DNA integration is largely determined by the enzymes available in the host cells during infection. In yeast, enzymes important for T-DNA integration have been identified. Enzymes involved in homologous recombination (Rad51, Rad52) play an important role in T-DNA integration by homologous recombination in yeast (Bundock *et al.* 1995; Attikum and Hooykaas 2003; Ohmine *et al.* 2016), while enzymes involved in non-homologous end-joining (Yku70, Yku80, Lig4) are essential for T-DNA integration by non-homologous recombination in yeast (Bundock and Hooykaas 1996; van Attikum *et al.* 2001). In plants, however, the proteins involved in non-homologous end joining (NHEJ) are not essential for T-DNA integration, but transformants can only be obtained when Polymerase θ, which is absent from yeast and fungi, is available (van Kregten *et al.* 2016).

Several chromatin components or chromatin-modifying enzymes were identified which play a role in stable T-DNA transformation in plants, such as histone H2A (Mysore *et al.* 2000) and H3 (Anand *et al.* 2007) and the histone deacetylases HDT1 and HDT2 (Crane and Gelvin 2007). In yeast histone, acetyltransferases (Gcn5, Ngg1, Yaf9 and Eaf7) and deacetylases (Hst4, Hda2 and Hda3), involved in chromatin modification, have also been identified as factors affecting AMT (Soltani *et al.* 2009). *ARP6* encoding an actin-related protein that is part of the SWR1 chromatin remodelling complex, negatively regulates AMT (Luo *et al.* 2015). Preliminary results in our group demonstrated that deletion of *ADA2* leads to enhanced transformation, when using a T-DNA that integrates by homologous recombination (Soltani 2009). The Ada2 protein is the chromatin-binding subunit of the SAGA (Spt–Ada–Gcn5 acetyltransferase) histone acetyltransferase (HAT) complex. This complex is involved in the post-translational modifications of histones that are crucial for chromatin-dependent functions and the regulation of numerous cellular processes in response to environmental cues (Sterner *et al.* 2002; for recent review, see Soffers and Workman 2020). Ada2 can interact with Gcn5 directly to increase its HAT activity which preferentially acetylates histone H3 and histone H2B (Grant *et al.* 1997; Hoke *et al.* 2008). The Spt constituent of the SAGA complex, consisting of the proteins Spt3 and Spt20, has a coactivator role in the recruitment of TATA-binding protein (Dudley *et al.* 1999). Ada2 is evolutionarily conserved among eukaryotes and has been described in several organisms, including *Arabidopsis* (Hark *et al.* 2009) and *Drosophila* (Muratoglu *et al.* 2003). In *Arabidopsis*, the orthologues of Ada2 physically associate with Gcn5 and enhance its HAT activity to regulate gene expression under environmental stress conditions such as cold, drought and salt stress (Hark *et al.* 2009). In 2009, an additional function of Ada2, independent of Gcn5, was identified in yeast. The novel role of Ada2 was to promote transcriptional silencing at telomeres through binding to Sir2 and to prevent the inward spread of heterochromatin regions (Jacobson and Pillus 2009).

In the present study, we investigated the role of *ADA2* in AMT in more detail. To this end, we analysed the effect of deletion of *ADA2* on T-DNA integration by homologous and non-homologous recombination and showed that deletion of *ADA2* resulted in an increased transformation efficiency for both targeted and random T-DNA integration.

Results and discussion

Increased AMT in yeast ada2Δ deletion mutants using T-DNA allowing integration by homologous recombination

Our preliminary results with the diploid yeast strain BY4743 suggested that the efficiency of AMT is increased in the *ada2Δ* deletion mutant (Soltani 2009). To investigate whether this is also the case in the isogenic haploid BY4741, we constructed an *ada2Δ* deletion mutant in BY4741 by replacing the *ADA2* coding sequence by an hygromycin resistance marker. Subsequently, the strain was transformed with the *Agrobacterium* strain LBA1100 carrying binary vector pRAL7100 allowing integration of *URA3* into the chromosomal *PDA1* locus by homologous recombination (Fig. 1a) (Bundock *et al.* 1995). As shown in Fig. 1a, the BY4741 *ada2Δ* deletion mutant had a four-fold increased transformation efficiency compared to the parental strain at frequencies of $4{\cdot}4 \pm 0{\cdot}6 \times 10^{-4}$ and $1{\cdot}1 \pm 0{\cdot}4 \times 10^{-4}$ (mean ± SEM, $n = 3$, $P = 0{\cdot}01$), respectively.

For our further studies, we preferred to use a marker-free *ada2Δ* mutant, as this would enable the use of a

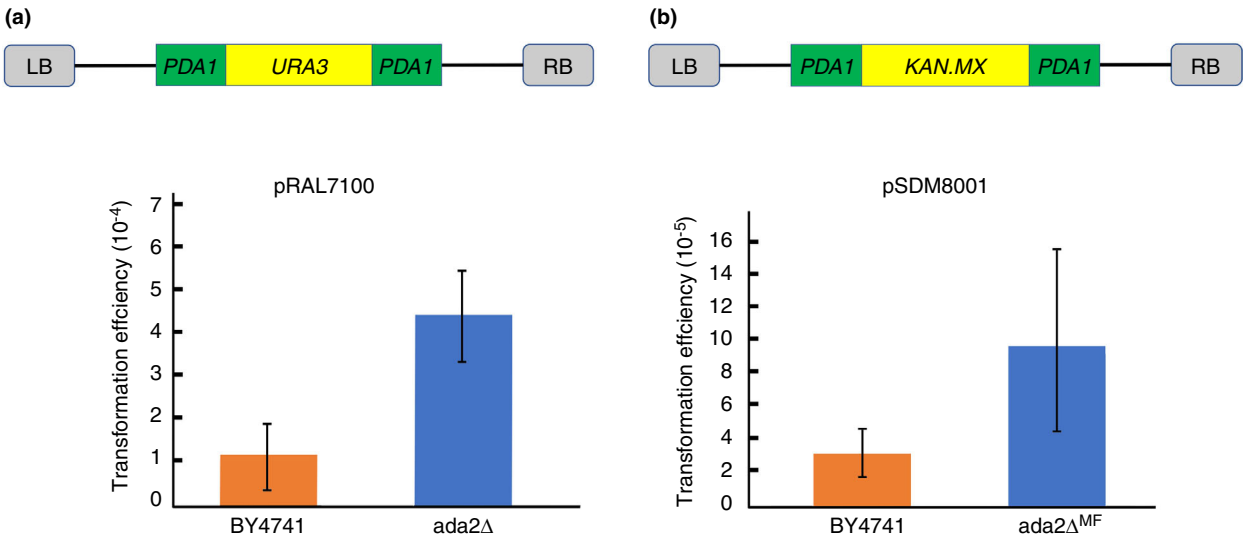

Figure 1 Increased *Agrobacterium*-mediated transformation of yeast *ada2Δ* deletion mutants by homologous recombination. (a) Transformation efficiency of yeast strain *ada2Δ* and its parental strain BY4741 upon co-cultivation with *Agrobacterium* strain LBA1100 harboring pRAL7100. The schematic diagram presents the structure of the T-DNA of pRAL7100. Error bars indicate the SEM of three independent assays. The difference is significant (P = 0·01). (b) Transformation efficiency of yeast strain ada2Δ^{MF} and its parental strain BY4741 both carrying plasmid YEp24 (to make growth conditions, the same as those used for other uracil prototrophic strains) upon co-cultivation with *Agrobacterium* strain EHA105 harbouring pSDM8001. The schematic diagram presents the structure of the T-DNA of pSDM8001. Error bars indicate the SEM of five independent assays. The difference is significant (P = 0·03). The different transformation frequencies of wild-type strain BY4741 shown in panels a and b may be due to the use of different *Agrobacterium* strains and/or different selection genes, but also partly to slightly different experimental conditions applied.

larger range of vectors for transformation. To this end, we deleted the *ADA2* coding region in BY4741 using the CRISPR-Cas technology resulting in strain *ada2Δ*^{MF}. This strain and BY4741 were co-cultivated with *Agrobacterium* strain EHA105 carrying plasmid pSDM8001 containing the KanMX cassette flanked by sequences allowing integration into the *PDA1* locus (Fig. 1b). G418-resistant transformants were selected and the transformation frequency was calculated. As shown in Fig. 1b, the transformation frequency of this *ADA2* deletion mutant was increased (3·1-fold) as well ($9{\cdot}7 \pm 2{\cdot}5 \times 10^{-5}$ *vs* $3{\cdot}1 \pm 0{\cdot}6 \times 10^{-5}$, mean ± SEM, n = 5, P = 0·03).

Increased AMT in yeast ada2Δ deletion mutants using T-DNA lacking sequences homologous to the yeast genome

Although homologous recombination is the predominant mechanism of T-DNA integration in yeast, integration via NHEJ is possible as well (Bundock and Hooykaas 1996; van Attikum *et al.* 2001). To investigate the effect of the *ada2Δ* deletion on T-DNA integration via NHEJ, we exploited *Agrobacterium* strain EHA105 harbouring plasmid pSDM8000. This plasmid contains a T-DNA with the KanMX cassette, but lacks homology with the BY4741 genome and has no yeast replication origin (Fig. 2a). As illustrated in Fig. 2a, *Agrobacterium* carrying pSDM8000 is able to transform BY4741, but at an extremely low frequency of $5 \pm 2 \times 10^{-6}$ (mean ± SEM, n = 10). Compared to the wild-type strain, the transformation efficiency for the *ada2Δ*^{MF} deletion mutant was significantly (P = 0·008) higher ($23 \pm 6 \times 10^{-6}$, mean ± SEM, n = 10). As shown in Fig. 2b, addition of a wild-type copy of *ADA2* on the centromeric plasmid pRS315 to the *ada2Δ*^{MF} deletion mutant restored the low wild-type transformation efficiency, indicating that the enhanced transformation of the *ada2Δ*^{MF} deletion mutant was due to the *ADA2* deletion and not to off-target effects of the CRISPR-Cas method. An additional copy of *ADA2* does not result in a further decrease in the transformation efficiency of BY4741.

To check whether the T-DNA is integrated into the yeast chromosome in the *ada2Δ*^{MF} strain and not present as an extrachromosomal structure, Southern blot analysis was done. To this end, the genomic DNA was isolated from 12 independent transformants obtained after co-cultivation of *ada2Δ*^{MF} or BY4741 with *Agrobacterium* strain EHA105 carrying pSDM8000. The DNA was digested with *Eco*RV and then hybridized to a probe containing the KanMX cassette. As pSDM8000 contains only two *Eco*RV sites outside the T-DNA region, the non-integrated plasmid and the integrated total plasmid will

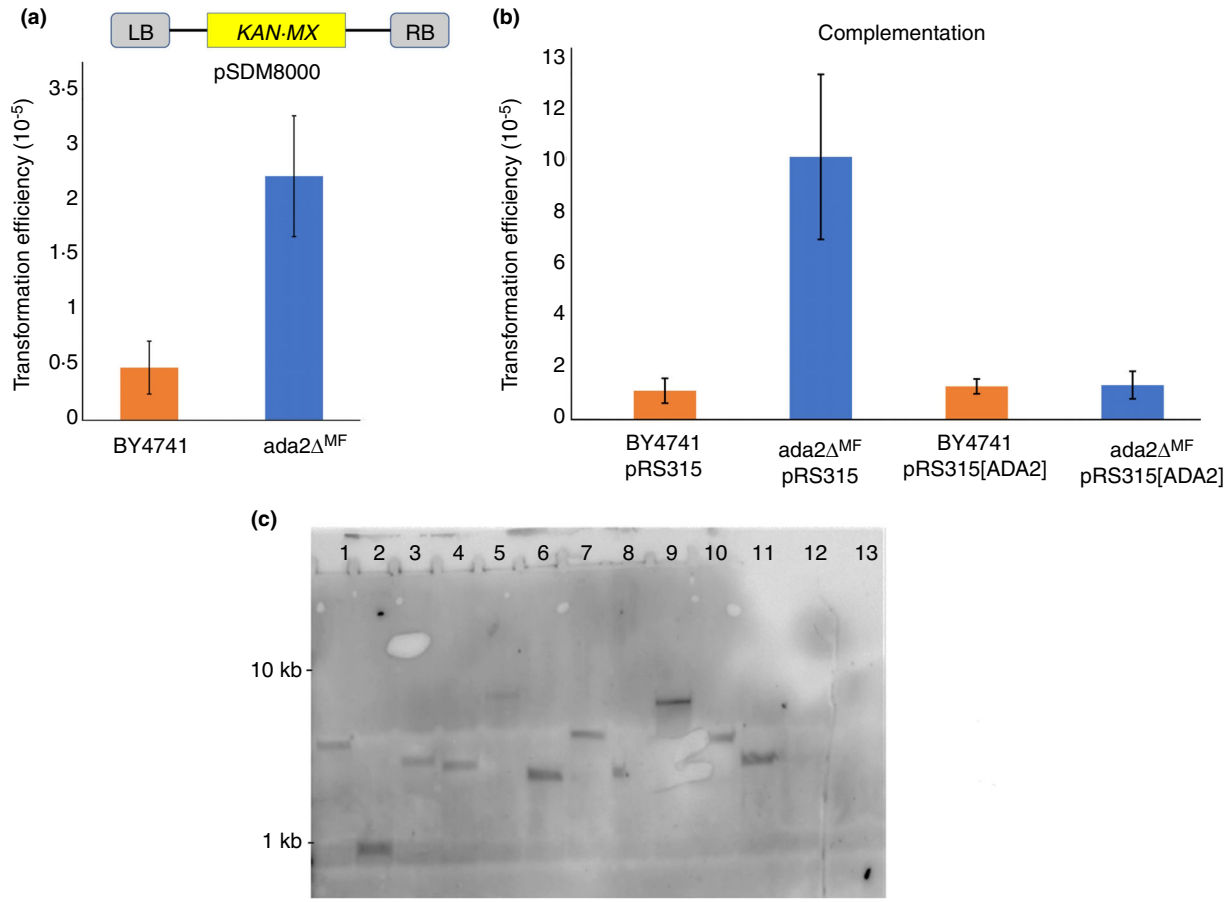

Figure 2 Increased *Agrobacterium*-mediated transformation of the yeast *ada2Δ*^{MF} deletion mutant by non-homologous recombination. (a) Transformation efficiency of yeast strain *ada2Δ*^{MF} and its parental strain BY4741 upon co-cultivation with *Agrobacterium* strain EHA105 harbouring pSDM8000. The schematic diagram presents the structure of the T-DNA of pSDM8000. Error bars indicate the SEM of 10 independent assays. The difference is significant (P = 0·008). (b) Complementation of the *ada2Δ*^{MF} deletion by a wild type copy of *ADA2*. The transformation efficiency of yeast strain *ada2Δ*^{MF} and its parental strain BY4741 carrying either pRS315 or pRS315[ADA2] upon co-cultivation with *Agrobacterium* strain EHA105 harbouring pSDM8000 is shown. Error bars indicate the SEM of three independent assays. For the experiments described in panel a and panel b slightly different experimental conditions were used, which prevent a direct comparison between frequencies seen in panel a and panel b. (c) Southern blot analysis of DNA isolated from 12 independent transformants of *ada2Δ*^{MF} (lanes 1–4 and 10–12) or its parental strain BY4741 (lanes 5–9) upon co-cultivation with *Agrobacterium* strain EHA105 harbouring pSDM8000. Lane 13 contains DNA isolated from untransformed *ada2Δ*^{MF}. DNA was digested with *Eco*RV and hybridized to a KanMX probe.

yield a 8·4 kb fragment hybridizing to the KanMX probe. Circularization of T-DNA will yield a circular DNA lacking *Eco*RV sites of approximately 1·5 kb. On the other hand, upon integration of T-DNA, the size of the chromosomal fragment containing the T-DNA and thus hybridizing to the KanMX probe is predicted to vary as it is dependent on the local presence of *Eco*RV sites in the genome. When integration occurs by NHEJ, it is expected that integration occurs at a variety of chromosomal positions and in different transformants fragments of different sizes are expected to hybridize to the probe. As shown in Fig. 2c, the probe indeed hybridizes to different *Eco*RV fragments in the DNA from the different transformants. For one of the transformants, hybridization to a fragment of about 1 kb which is smaller than the T-DNA (*c.* 1·5 kb) was found (Fig. 2c, lane 2), possibly caused by integration of a part of the T-DNA. No hybridization was found for DNA isolated from the untransformed *ada2Δ*^{MF} strain (Fig. 2c, lane 13). These results indicate that the T-DNA was integrated at different sites into the chromosomal DNA of the transformants and therefore likely had occurred by NHEJ.

Increased double-strand breaks in the ada2Δ deletion mutant

Ada2-dependent histone acetylation has been shown to be involved in double-strand break repair (Muñoz-Galván

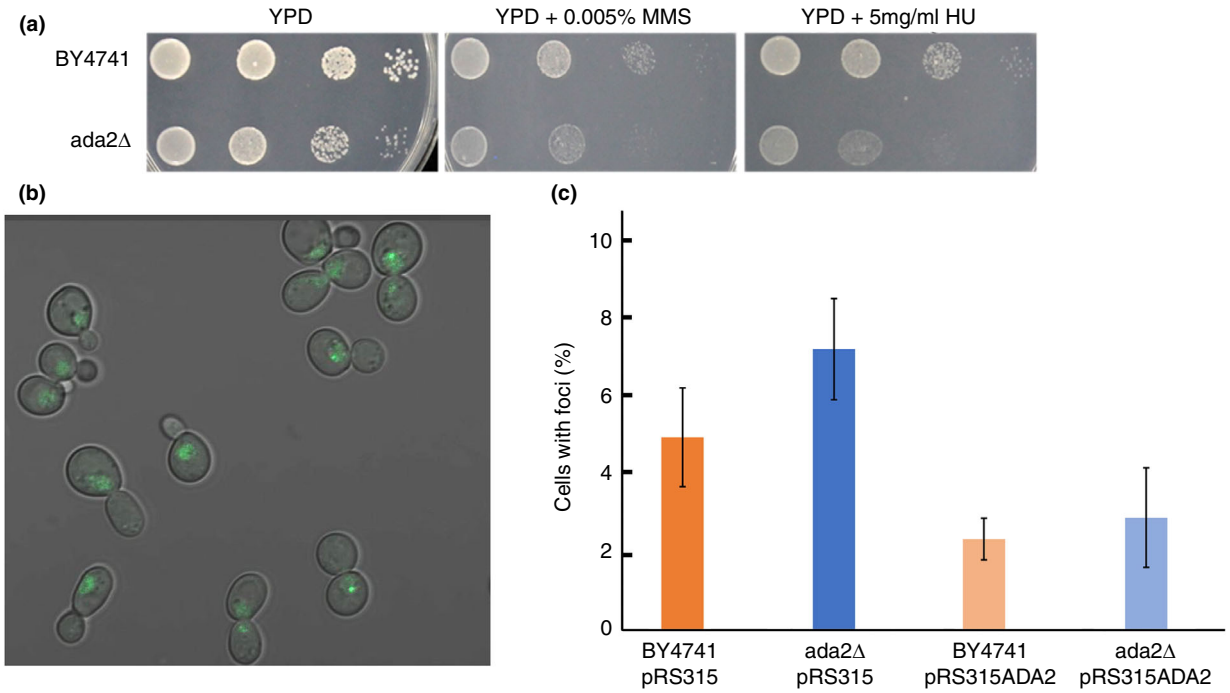

Figure 3 The *ada2Δ* deletion mutant is more sensitive to the DNA damaging agents methyl methane sulfonate (MMS) and hydroxyurea (HU) and has an increased number of double-strand breaks. (a) Both wild-type BY4741 and *ada2Δ* deletion mutant were tested on yeast extract-peptone-dextrose (YPD) plates with two commonly used DNA damaging agents, MMS and HU. Yeast cells were serially diluted and spotted onto the plates. The photos were taken after 3 days and representative results of three independent experiments are shown. (b) The Rad52 protein was marked by GFP to visualize double-strand breaks in *ada2Δ* cells. The DNA repair foci were observed using confocal microscopy. (c) The percentage of cells showing DNA repair foci is shown for both BY4741 and the *ada2Δ* deletion mutant carrying pRS315 or pRS315[ADA2]. The percentage of cells with foci is the average with SEM of the percentages determined after seven independent cultivations. For each strain at least 2100 cells were observed.

et al. 2013). As double-strand breaks can promote T-DNA integration, we investigated the sensitivity of the *ada2Δ* deletion mutant for DNA damaging agents. The DNA alkylating agent methyl methane sulfonate (MMS) induces double-strand breaks during replication and hydroxyurea (HU) is a potent inhibitor of the enzyme ribonucleotide reductase in S-phase and leads to stalling of DNA replication. Survival viability was estimated by plating serial dilutions of cultures of wild type and *ada2Δ* deletion mutant cells on YPD plates containing MMS or HU. As demonstrated in Fig. 3a, the deletion of *ADA2* enhanced the sensitivity to the DNA damaging agents MMS and HU. To obtain further evidence for the presence of relatively more chromosomal DNA damage in *ada2Δ* mutants, we analysed Rad52 foci formation (Fig. 3b). Rad52 is a master regulator protein of DNA repair via homologous recombination and Rad52 is recruited to double-strand breaks, which can be seen as foci, when using GFP-tagged Rad52 (Lisby *et al.* 2001). As shown in Fig. 3c, in 4·7% of the BY4741 cells carrying the empty pRS315 vector Rad52-GFP foci were observed. This percentage increased to 6·8% for *ada2Δ* cells carrying the empty pRS315. In *ada2Δ* cells carrying a wild-type copy of *ADA2* in pRS315 (pRS315[ADA2]), this percentage was significantly ($P = 0{\cdot}02$) down to 2·1, indicating that deletion of *ADA2* results in an increased number of double-strand DNA breaks. Addition of pRS315 [ADA2]) to the BY4741 control also decreased the number of Rad52 foci (2·2 *vs* 4·7%; $P = 0{\cdot}04$), suggesting that *ADA2* overexpression results in a decreased number of double-strands breaks.

It is still unknown why deletion of *ADA2* results in an increased AMT efficiency. Chromatin modifications play a crucial role in DNA repair mechanisms which are exploited to facilitate T-DNA integration. Several observations have been described and reviewed (Magori and Citovsky 2011; Shilo *et al.* 2017), indicating that the histone acetylation balance is important for T-DNA integration even though its molecular basis remains unclear. *ADA2* is a component of HAT complexes related to chromatin modifications (Berger *et al.* 1992; Grant *et al.* 1997; Balasubramanian *et al.* 2002). Another possible explanation is that increased AMT efficiency is the consequence of altered expression of certain genes directly or indirectly

Table 1 Plasmids used in this study

Plasmid	Specifications	Source/Reference
pAG32	Plasmid with the *hph* gene encoding hygromycin B phosphotransferase	Goldstein and McCusker (1999)
pYM27	Plasmid used as PCR template for C-terminal eGFP tagging using the *kanMX* marker	Janke *et al*. (2004)
pML104	Plasmid for expression of Cas9 and contains guide RNA expression cassette. *URA3* selection marker	John Wyrick (Addgene plasmid # 67638) Laughery *et al*. (2015)
pML104 [ADA2 disr] (pSDM3793)	Plasmid for deletion of *ADA2* by the CRISPR-Cas technology	This study
YEp24	Yeast episomal cloning vector with a *URA3* marker	Carlson and Botstein (1982)
pRS315	Yeast centromeric plasmid with a *LEU2* marker	Sikorski and Hieter (1989)
pRS315 [ADA2] (pSDM3792)	pRS315 containing a 2026 bp fragment containing *ADA2* under control of its native promoter and terminator	This study
pRAL7100	*Agrobacterium* binary vector with *URA3* selectable marker and *PDA1* flanking sequence	Bundock *et al*. (1995)
pSDM8000	*Agrobacterium* binary vector with *kanMX* selectable marker	Attikum and Hooykaas (2003)
pSDM8001	*Agrobacterium* binary vector with *kanMX* selectable marker and *PDA1* flanking sequence	Attikum and Hooykaas (2003)
pUG6	Plasmid containing the KanMX gene disruption cassette	Güldener *et al*. (1996)

regulated by *ADA2*. The expression of approximately 2·5% of all yeast genes was found to be affected at least twofold in an *ada2Δ* deletion mutant (Hoke *et al*. 2008). It can be speculated that the increased AMT efficiency of the *ada2Δ* deletion mutant could be the consequence or an indirect influence caused by altered regulation of certain genes involved in the integration process. Besides such indirect roles of *ADA2* in gene expression or chromatin structure, we showed there is more DNA damage in an *ada2Δ* deletion mutant as revealed by an increased number of Rad52 foci (Fig. 3). Our results are in line with the results obtained in a large-scale screen for deletion mutants with an increased percentage of cells with one or more spontaneous Rad52-YFP foci (Alvaro *et al*. 2007). In the initial screen, 11% of the ada2Δ cells contained foci, compared to 5% of the wild-type cells. Furthermore, it has been shown that an Ada2 homologue in plants plays a role in double-strand break repair (Lai *et al*. 2018). Due to the absence of *ADA2*, there may be either more DNA damaging events in the cell or this damage may be repaired less efficiently, thus providing more available sites for T-DNA integration.

AMT of some yeasts and fungi occurs only with a very low efficiency (Idnurm *et al*. 2017). To improve this efficiency, it may be worthwhile to investigate whether disruption of *ADA2* homologues in such organisms has a similar effect as we observed in the yeast *S. cerevisiae*. As disruption of *ADA2* has additional effects, the use of a system that temporary represses *ADA2* expression may be of interest.

Materials and methods

Agrobacterium strains and growth conditions

Agrobacterium tumefaciens strains LBA1100 (Beijersbergen *et al*. 1992) and EHA105 (Hood *et al*. 1993) were used; both strains are derivatives of strain C58, in which the natural Ti plasmid is replaced by a *vir* helper plasmid. Binary vector plasmids were introduced into *Agrobacterium* by electroporation (Dulk-Ras and Hooykaas 1995). *Agrobacterium tumefaciens* strains were grown and maintained at 28°C in LC medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 8 g l^{-1} NaCl) containing carbenicillin (100 μg ml^{-1}), kanamycin (100 μg ml^{-1}) or rifampicin (20 μg ml^{-1}) when required.

Yeast strains and growth conditions

Saccharomyces cerevisiae strains used in this study are BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann *et al*. 1998), ada2Δ (LBY1135) (BY4741 *ada2Δ::hphMX4*) (this study), ada2Δ^{MF} (LBY1148) (BY4741 *ada2Δ*) (this study), BY4741 Rad52-GFP (LBY1137) (BY4741 *RAD52-GFP-kanMX4*) (this study) and ada2Δ Rad52-GFP (LBY1138) (BY4741 *ada2::hphMX4 RAD52-GFP-kanMX4*) (this study). Plasmids used in this study are shown in Table 1 and primers in Table 2. The *ada2Δ* deletion in BY4741 containing the HphMX4 cassette was constructed using the PCR-mediated one-step gene disruption method. The disruption cassette was obtained by PCR with the Ada2-Fw and Ada2-Rev primers using pAG32 as template and hygromycin-resistant transformants were selected. Correct integration was checked by PCR using the primers Ada2-ctrl-Fw and Ada2-ctrl-Rev. For C-terminal labelling of Rad52 with GFP, a DNA fragment was used obtained by PCR with the primers Rad52-gfp-Fw and Rad52-gfp-Rev and plasmid pYM27 as template.

Table 2 Primers used in this study

Ada2-Fw	TAAAATATCAGCGTAGTCTGAAAATATATACATTAAGCAAAAAGACAGCTGAAGCTTCGTACGC
Ada2-Rev	ATAATAACTAGTGACAATTGTAGTTACTTTTCAATTTTTTTTTTGCCGCGGCCGCATAGGCCAC
Ada2-Ctrl-Fw	ACGACCTCTGAGAAAACGA
Ada2-Ctrl-Rev	GGTCCCTTTATGACTTGGC
Rad52-gfp-Fw	AGAGAAGTTGGAAGACCAAAGATCAATCCCCTGCATGCACGCAAGCCTACTCGTACGCTGCAGGTCGAC
Rad52-gfp-Rev	AGTAATAAATAATGATGCAAATTTTTTATTTGTTTCGGCCAGGAAGCGTTTCAATCGATGAATTCGAGCTCG
Ada2-repair-1	ACCCTCCATTTTCGATAAAATATCAGCGTAGTCTGAAAATATATACATTAAGCAAAAAGACAAAAAAAAAATTG AAAAGTAACTACAATTGTCACTAGTTATTATTGGCCAAGTCATAAA
Ada2-repair-2	TTTATGACTTGGCCAATAATAACTAGTGACAATTGTAGTTACTTTTCAATTTTTTTTTTGTCTTTTTGCTTAATGTATATATTTTCA GACTACGCTGATATTTTATCGAAAATGGAGGGT
P-gRNA-5	GGGAACAAAAGCTGGAGCTCC
Ada2-guide-Rev	CTAGCTCTAAAACTTACGGGACCTTCAGCTTCAGATCATTTATCTTTCACTGCGGAG
HindIII-ADA2-Fw	AAAAAGCTTTTTTATCTGCTTTTTTCTTTATCTATTTATTC
PstI-ADA2-Rv	AACTGCAGATGCGGTACTGTACATTTTATAAATG
KANMX-Fw	CCAGCTGAAGCTTCGTACGC
KANMX-Rev	CATAGGCCACTAGTGGATCTG

For the marker-free deletion of *ADA2* by the CRISPR-Cas technique, BY4741 was co-transformed with 250 ng of plasmid pML104[ADA2 disr] and 1 μg of the repair fragment and transformants were selected for uracil prototrophy. The repair fragment was obtained by annealing oligos Ada2-repair-1 and Ada2-repair-2. Transformants are expected to have the required deletion but they still contain the pML104[ADA2 disr] plasmid. The transformants were streaked on a plate containing 5-fluoro-orotic acid (1 mg ml^{-1}) and uracil in addition to methionine, histidine and leucine to select for cells that had lost the plasmid. After incubation for 5 days at 30°C, colonies were analysed by restreaking on plates containing or lacking uracil. Uracil auxotrophic transformants were selected and DNA was isolated. The *ADA2* deletion was analysed by PCR using primers Ada2-ctrl-Fw and Ada2-ctrl-Rev, followed by sequencing of the PCR fragments.

The PCR products and plasmids were transferred to yeast cells using the lithium–acetate transformation protocol (Gietz *et al.* 1995). Yeast was grown at 30°C in yeast extract–peptone–dextrose (YPD) medium supplemented, when required, with the appropriate antibiotic G418 (200 μg ml^{-1}) or hygromycin (200 μg ml^{-1}) or in selective minimal yeast (MY) medium (Zonneveld 1986) supplemented with appropriate nutrients. For spot plate assays, cultures were adjusted to an OD_{620} of 0·1 after growth to saturation in liquid YPD. Then, 10-fold serial dilutions were made and aliquots of 5 μl were spotted on YPD, on YPD containing 5 mg ml^{-1} HU and on YPD containing 5 mg ml^{-1} MMS.

Plasmid construction

The centromeric plasmid pRS315[ADA2] was constructed by ligation of a 2026 bp PCR fragment containing the *ADA2* promoter, coding and terminator sequences into pRS315 after digestion with *Hind*III and *Pst*I. The PCR fragment was obtained using the primers HindIII-ADA2-Fw and PstI-ADA2-Rv and BY4741 genomic DNA as template.

For the marker-free deletion of *ADA2*, the CRISPR-Cas technology was used. Plasmid pML104 was digested with *Sac*II and *Swa*I and the digested vector was isolated by gel electrophoresis. The guide RNA fragment was obtained by PCR on undigested pML104 using primers P-gRNA-5 and Ada2-guide-Rev. The guide fragment was digested with *Sac*II and ligated in pML104 digested with *Sac*II and *Swa*I yielding plasmid pML104[ADA2 disr].

Agrobacterium-mediated transformation efficiency test

AMT efficiency was determined as described (Bundock *et al.* 1995) with some modifications. First, *S. cerevisiae* strains and *Agrobacterium* were cultured overnight at 30 and 28°C, respectively, under continuous agitation and with the appropriate nutrition or antibiotic selection. The following day, the *Agrobacterium* cells were washed and re-suspended to an OD_{600} of 0·25 in induction medium (IM) with added glucose (10 mmol l^{-1}), acetosyringone (0·2 mmol l^{-1}) and the appropriate antibiotics, and incubated for another 6 h at 28°C. Meanwhile, yeast cultures were diluted to an OD_{620} of 0·1 and incubated in either liquid YPD or MY (when the yeast contained a plasmid) medium. After 6 h, the yeast cells were washed and re-suspended in 0·5 ml of IM, to a final OD_{620} of 0·4–0·6 and mixed with an equal volume of *Agrobacterium* cells and vigorously vortexed. Subsequently, 100 μl of the mixture was pipetted onto sterile nitrocellulose filters laid on IM plates supplemented with histidine, leucine and methionine. Once filters were dry, plates were incubated at 21°C for 6–7 days. After co-cultivation, the cells on each filter were resuspended and then spread onto solid

medium containing cefotaxime (200 μg ml^{-1}) with or without G418 (200 μg ml^{-1}). Finally, after a 3-day incubation at 30°C, colonies were counted. Yeast AMT efficiency was calculated by dividing the number of colonies on the selective plate by the number of colonies on the non-selective plates.

Southern blotting

Two micrograms of isolated yeast DNA were digested with *Eco*RV. The digested DNA was separated on a 0·7% (w/v) agarose gel and transferred onto a Nylon membrane (positively charged; Roche, Mannheim, Germany) by capillary blotting. The blot was hybridized to a KanMX probe at 68°C, labelled using the PCR DIG Labeling Mix (Roche), pUG6 as template and the primers KANMX-Fw and KANMX-Rev. Bound probe was detected using anti-digoxigenin-alkaline phosphatase and the CDP-star substrate (Roche).

Confocal microscopy

For microscopy, a Zeiss Axioscan confocal microscope with a 63× oil objective was used. GFP was detected using an argon laser of 488 nm and a band-pass emission filter of 505–600 nm. Images were processed with ImageJ (ImageJ National Institute of Health) (Schindelin *et al.* 2012).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contributions

M.R.R. and S.S. performed the experiments and contributed to the design of the study and writing the manuscript. I.P. performed the experiments and participated in data analysis. P.J.J.H. contributed to the design of the study and writing the manuscript and provided the funding support. G.P.H.v.H. supervised the study and contributed to the design of the study and writing the manuscript. All authors read and approved the submission of the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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