MINI-REVIEW

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Yeast-based genotoxicity tests for assessing DNA alterations and DNA stress responses: a 40-year overview

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Abstract

By damaging DNA molecules, genotoxicants cause genetic mutations and also increase human susceptibility to cancers and genetic diseases. Over the past four decades, several assays have been developed in the budding yeast *Saccharomyces cerevisiae* to screen potential genotoxic substances and provide alternatives to animal-based genotoxicity tests. These yeast-based genotoxicity tests are either DNA alteration-based or DNA stress-response reporter-based. The former, which came first, were developed from the genetic studies conducted on various types of DNA alterations in yeast cells. Despite their limited throughput capabilities, some of these tests have been used as short-term genotoxicity tests in addition to bacteria- or mammalian cell-based tests. In contrast, the latter tests are based on the emergent transcriptional induction of DNA repair-related genes via activation of the DNA damage checkpoint kinase cascade triggered by DNA damage. Some of these reporter assays have been linked to DNA damage-responsive promoters to assess chemical carcinogenicity and ecotoxicity in environmental samples. Yeast-mediated genotoxicity tests are being continuously improved by increasing the permeability of yeast cell walls, by the ectopic expression of mammalian cytochrome P450 systems, by the use of DNA repair-deficient host strains, and by integrating them into high-throughput formats or microfluidic devices. Notably, yeast-based reporter assays linked with the newer toxicogenomic approaches are becoming powerful short-term genotoxicity tests for large numbers of compounds. These tests can also be used to detect polluted environmental samples, and as effective screening tools during anticancer drug development.

Keywords *Saccharomyces cerevisiae* · Genotoxicity test · DNA alteration · Mutagen · Anticancer drug · Reporter assay

Introduction

DNA damage from various mutagens, including genotoxic chemicals, leads to cell death or, in humans, increased susceptibility to cancers and inherited disorders via accumulated genetic mutations (Friedberg et al. 2005). Despite the serious biological effects of mutagens on the individuals exposed to them and their offspring, DNA damage, unlike cytotoxic damage, is hardly detected by our sensing systems. In modern industrialized societies, a large number of newly generated chemicals are disposed of in the environment, and some are likely taken into our bodies through food supplements,

pharmaceuticals, cosmetics, or insecticide residues. Such chemicals might have potential carcinogenicity in mammals. Thus, sensitive bioassays have been developed to determine the genotoxicities (that is, the ability to cause DNA damage) of potential genetic risks hidden in the environment and in chemicals themselves.

To accurately assess chemical carcinogenicity in mammals, in vivo rodent carcinogenicity tests are used, the results of which are translated to humans. However, the results of such tests can be slow to obtain (on average 12–16 weeks), are costly, have ethical issues associated with them, and have high species variability. Since the 1970s, several bacteria-based bioassays have been developed to screen chemicals for potential genotoxicity as alternatives to animal-based tests (Reifferscheid and Buchinger 2010). The Ames test is based on monitoring the reverse mutations caused by mutagens in *Salmonella* mutant strains (Ames et al. 1973). This test has been universally used for this purpose, but some Ames-negative compounds are carcinogenic to animals, and some Ames-positive compounds are not. These tests in prokaryotic

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cells are easy to handle, inexpensive, and scalable to high-throughput formats and provide rapid results. However, as noted above, the results obtained from bacterial testing should be carefully considered for their relevance to eukaryotic organisms. This is because of the differences in the systems used for drug metabolism, membrane transport, and DNA repair. Although several mammalian cell-based tests (for example, the micronucleus and comet tests) have been developed and used in place of animal tests, they are laborious, require special equipment and training, and are expensive. Unicellular eukaryote-based tests carry a distinct advantage over bacteria- and animal-based tests in that they are both highly relevant for genotoxicity assessment of chemicals and environmental samples and are relatively easy to apply.

The budding yeast *Saccharomyces cerevisiae*, a representative unicellular eukaryote, is used for the production of foods and beverages, as well for molecular genetics research. *S. cerevisiae* offers three advantages for genotoxicity testing. First, yeast cells grow rapidly in growth medium, and, like bacteria, they are easily handled and genetically manipulated. Yeast cells have tough cell walls that are made of two layers. The outer layer, which is composed of highly glycosylated mannoproteins, limits the permeability of the cell wall due to its highly negatively charged groups and acts an efficient barrier to metal ions and organic compounds. The inner layer is made up of β-glucan and chitin and is largely associated with the mechanical strength of the cell walls (Klis et al. 2002). Because of their cell walls, yeast cells are tolerant to various physicochemical stresses, making them excellent biosensing tools. Second, as a eukaryote, *S. cerevisiae* shares many conserved biochemical pathways with humans, which is an important consideration when assessing the potential genotoxicity of a compound towards humans. Finally, as a model organism, *S. cerevisiae* offers many advantages for genotoxicity studies. For example, the biological functions of a large fraction of the 6000 genes in the yeast genome have been characterized in detail, and budding yeast is currently the best eukaryotic model for synthetic biology. By virtue of these advantages, several yeast-based assays have been developed as whole-cell biosensors for assessing ecotoxicity, an area that also includes assessment of genotoxicity (Baronian 2004; Jarque et al. 2016; Svobodová and Cajthaml 2010). Because yeast cells respond to DNA damage in a similar manner to mammalian cells (described later on), yeast-based tests can detect a wider range of genotoxicants than bacteria-based tests and are suitable for assessing potential genotoxicity to mammals. In this review, I provide an overview of the progress made in *S. cerevisiae*-based genotoxicity tests over the past four decades, and newly classify previous and current yeast-based tests into two types: (1) DNA alteration-based genotoxicity tests and (2) transcriptional responses to DNA stress-based tests (Fig. 1). In this context, I briefly describe the principles underlying the use of yeast-based genotoxicity tests, their advantages and limitations, and the technical improvements that have been made to reporter-based assays, followed by a discussion of the future of the genotoxicity field.

Yeast genotoxicity tests based on DNA alterations

Damage to genomic DNA, which can be of various types, is caused by genotoxic agents. These DNA lesions, which are detected by DNA damage checkpoint proteins in eukaryotes, are restored primarily by DNA repair proteins in a DNA damage-type-dependent pathway (Friedberg et al. 2005). Parts of the unrepaired or incorrectly repaired lesions generate structural alterations in the DNA. These can include point mutations, gene conversions, and DNA deletions. Therefore, the DNA alterations induced by genotoxicants can be used as markers to indicate the genotoxicities of these compounds. Several genotoxicity assays have been developed based on mutagen-induced DNA alterations in *S. cerevisiae* (Brusick and Mayer 1973; Moustacchi 1980). Along the same lines as the Ames test, the induction of forward and reverse mutations in auxotrophic genes has also been utilized in yeast-based genotoxicity tests. Other types of DNA alterations (including gene conversion, mitotic cross over, deletion, chromosomal malsegregation, and DNA strand breakage), as well as transposon induction and DNA damage to mitochondrial DNA or transgenes, have also been used as genotoxicity indicators. A chemical's genotoxicity is usually judged as positive after reproducible, dose-dependent increases in these indicators are detected in the treated yeast. In contrast, a compound is considered a non-mutagen if no dose-dependent increases in these indicators are observed after repeated experiments. It is recommended that a toxic dose range experiment is performed prior to the tests to determine the appropriate dose ranges of test compounds and their toxicity thresholds. Appropriate positive and negative (solvent) control compounds should be used in the tests to monitor for significant background. The genotoxicity of a tested chemical can be evaluated by comparing the test results with those of well-known genotoxicants (positive controls). Most of these short-term yeast-based tests were developed between the 1970s and the 1990s, and their usefulness and technical limitations are described briefly in the following sections.

Mutagenesis tests

The reverse mutation of auxotrophic genes in yeasts is used as an indicator of genotoxicity. In this assay, 10^6–10^7 chemical-treated tester cells are spread per agar plate containing auxotrophic selection medium, and the reversion frequency is determined by counting the number of revertant colonies. A

Fig. 1 Yeast-based genotoxicity tests. Yeast-based genotoxicity tests are classified as two types: DNA alteration-based tests (upper part) and DNA stress response-based tests (lower part). The genotoxic effects and the representative detection methods used for each type of test are shown
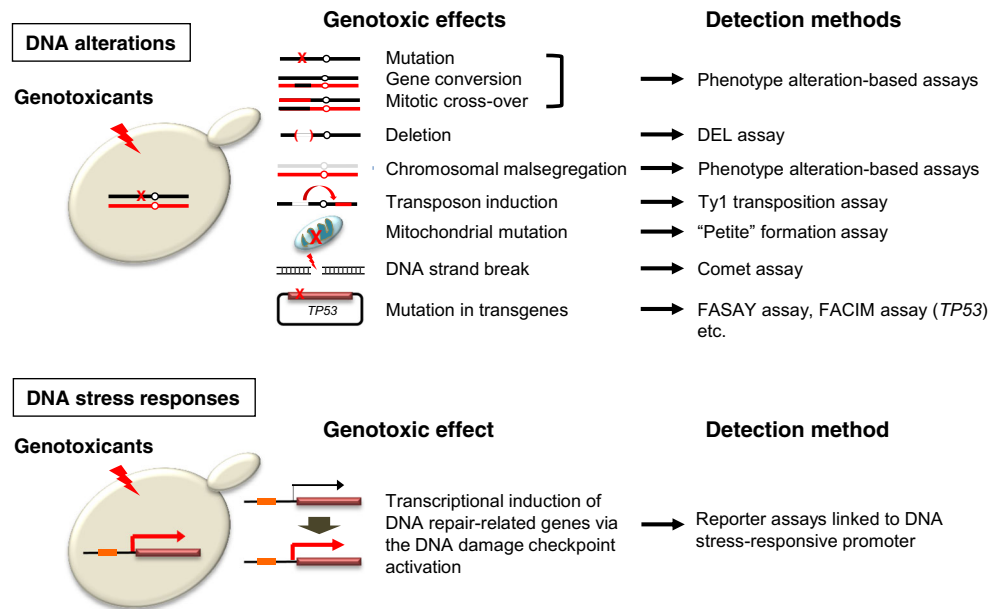
reproducible dose-dependent increase in the reversion frequency in the chemical-treated tester cells is judged to be positive in its genotoxicity. Haploid strains with mutated auxotrophic genes have been used as tester strains. For example, XV185-14C is a representative strain that contains six mutated auxotrophic alleles, which enable mutation frequency monitoring in this strain (Shahin and von Borstel 1976). Diploid strains with mutated loci are also often used for genotoxicity tests because they can simultaneously detect recombination-mediated DNA alterations (discussed in the next section). For example, mutagenicity at the *ilv1-92* locus in strain D7 can be assayed via the emergence of isoleucine-independent colonies.

Gene conversion, mitotic cross over, and deletion tests

Gene conversion, mitotic cross over, and DNA deletions frequently occur via DNA recombination-mediated repair of DNA damage, suggesting these occurrences are suitable for use as genotoxicity indicators. Diploid strains with mutated alleles of auxotrophic genes are used for the DNA recombination tests, as reversion to prototrophy in a heteroallelic diploid is caused most frequently by gene conversion rather than by reverse mutation in the mutated allele. The genotoxicity of a particular chemical is judged as positive based on a reproducible, dose-dependent increase in the frequency of prototrophic colonies. Several well-defined yeast diploid strains (for example, D4, Bronzetti et al. 1978; D5, Ferguson and Turner 1988a; and D7, Zimmermann et al. 1975) were established in the 1970s for detecting mutagen-induced DNA recombination events. In particular, the D7 strain, which is used for detecting point mutations, gene conversion events, and mitotic cross

overs, has been used for assessing the genotoxicities of bleomycin (Hannan and Nasim 1978) and paraquat (el-Abidin Salam et al. 1993), as well as various insecticides (Bianchi et al. 1994; Miadoková et al. 1992; Stehrer-Schmid and Wolf 1995), pollutants (Bronzetti et al. 1978; Frassinetti et al. 2011), and environmental samples (Buschini et al. 2001; Giorgetti et al. 2011; Magdaleno et al. 2008; Pellacani et al. 2006). Schiestl et al. developed the RS112 diploid strain, which has a deletion mutation in one copy of the *HIS3* gene while the second *HIS3* copy is disrupted by the insertion of plasmid DNA containing an internal fragment of the *HIS3* gene. Once the plasmid is deleted via intrachromosomal recombination, the RS112 cells can grow in medium lacking histidine. The genotoxicity of the agent being tested is judged as positive if there is a dose-dependent increase in the formation of histidine prototrophic colonies in the treated tester cells. This yeast "DEL assay" has successfully detected the induction of intrachromosomal deletions by various genotoxic compounds (Brennan et al. 1994; Kirpnick et al. 2005; Schiestl 1989; Schiestl et al. 1989), suggesting its usefulness for genetic toxicity assessments (Ku et al. 2007). Despite the development of a microplate-based DEL assay (Hontzeas et al. 2007), the mutagenesis tests discussed in the previous section and these recombination-based tests both require laborious plating work using different auxotrophic selection media to test genotoxicity in a sample.

Chromosomal malsegregation assays

Chromosomal malsegregation (otherwise known as chromosome non-disjunction) and the resultant aneuploidy in mitosis and meiosis increase susceptibility to cancer and congenital disorders, respectively. Assays for screening

chromosomal malsegregation using special yeast diploid strains have been reported (Resnick et al. 1986). For example, chromosome XV of strain D61.M contains a homoallelic *ade2-40* gene, and each chromosome VII contains *TRP5*, *ade6*, *leu1* and *cyh*^{R}*2* and *trp5*, *ADE6*, *LEU1*, and *CYH2* genes, respectively. This strain forms red colonies and is sensitive to cycloheximide. Loss of the chromosome VII copy with *trp5* results in expression of *ade6* (white colonies, requiring adenine supplementation) and *cyh*^{R}*2* mutations (cycloheximide resistant), and such cells form white colonies on medium containing cycloheximide and adenine. The increased frequency of such colonies in the chemical-treated yeast is primarily used to assess the ability of the chemical to cause chromosomal malsegregation. Although more than 100 compounds have been tested using the D61.M strain between 1984 to 1990, it is noteworthy that the induction of chromosomal malsegregation by some chemicals is likely to be caused by their interactions with targets other than DNA, such as tubulin or membrane systems (Albertini and Zimmermann 1991). In 2008, Schafer et al. developed a yeast diploid strain for simultaneous detection of chromosome non-disjunction as well as mutations, mitotic cross overs, and gene conversion events (Schafer et al. 2008).

DNA strand break (comet) assays

The single-cell electrophoresis assay, or the comet assay using mammalian cells, was developed by Singh et al. (Singh et al. 1988) and has been widely used to detect DNA strand breaks caused by genotoxic agents. Briefly, sample cells are suspended in molten low-melting-point agarose and the suspension is cast onto a slide glass. The cells in the gel are lysed and subjected to electrophoresis, and the DNA is stained with fluorescent dye, after which the pattern of migrated DNA (with the shape of a comet) is visualized under a UV microscope. The intensity of the comet tail relative to its head reflects the number of DNA strand breaks induced by the agents being tested and is used for judging their genotoxicities. Yeast comet assays have been used to assess the genotoxicity of various agents (Azevedo et al. 2011; Lah et al. 2004; Miloshev et al. 2002; Nemavarkar et al. 2004; Rank et al. 2009). Recently, a yeast comet-FISH technique was developed to detect DNA strand breaks at the chromosomal level (Lewinska et al. 2014). Yeast comet assays must be performed under a microscope after carefully treating the cells to disintegrate their cell walls and enabling them to be embedded in agarose.

Mitochondrial mutation assay

S. cerevisiae cells with dysfunctional mitochondria can survive in a respiratory-deficient state and form small-sized colonies called "petites" on agar medium. Some genotoxic chemicals, such as the intercalating agent ethidium bromide, strongly induce "petite" formation. An assay based on this phenotype has been used to assess the genotoxicity of anticancer drugs, including DNA damaging agents (Ferguson and Turner 1988b). However, some chemicals' abilities to induce the "petite" phenotype are not consistent with mutagenicity in the chromosomes themselves. For example, strong "petite" inducers such as ethidium bromide, methotrexate, and 5-fluorouracil are not very active mutagens (Ferguson and Turner 1988b). Therefore, the "petite" phenotype may reflect agent-specific genotoxic and/or cytotoxic effects.

Transposon induction test

Ty1 is the most abundant class of retrotransposable elements in yeast. Ty1 elements can potentially replicate via an RNA intermediate and generate a DNA copy by reverse transcription, and then randomly integrate into the yeast genome (termed Ty1 transposition) to cause genome instability. Although the rate of spontaneous Ty1 transposition is very low, transposition is strongly induced under certain stress conditions, such as DNA damage. Pesheva and colleagues developed a Ty1 transposition assay for sensing the DNA damage caused by chemicals and for detecting genotoxins in polluted soil samples (Pesheva et al. 2005; Pesheva et al. 2008). In these studies, Ty1 transposition was induced in a permeable mutant tester strain (DG114ts1) upon exposure to several known mutagens, certain procarcinogens, polluted soil samples treated with S9 extracts (prepared from rodent livers containing drug metabolizing enzymes as described later), and three compounds previously shown to be negative in the DEL assay. The genotoxicities of the tested agents are judged as positive if there is an increased frequency of histidine prototrophic colonies caused by Ty1 transposition. Despite the usefulness of the transposon induction test, measuring the mutation frequency of a compound via several fluctuation tests demands a lot of plating work, making sample throughput a significant limitation.

Reporter assays for mutation analysis

Two types of yeast-based reporter assays have been developed. The first type analyzes the mutation spectrum of target genes, such as *SUP4*-o (Pierce et al. 1987) and *TP53* (Billet et al. 2010; Cachot et al. 2004; Inga et al. 1997; Murata et al. 1997; Paget et al. 2008a, b), while the second type detects mutations in microsatellite DNA (Marden et al. 2006). The tumor suppressor gene, *TP53*, has often been used as a target gene because of its importance in carcinogenesis, and several reporter assays based on this gene have been established, including the FACIM (Cachot et al. 2004) and FASAY (Billet et al. 2010; Paget et al. 2008a, b) assays. In the FACIM assay,

plasmid DNA containing the wild-type *TP53* gene is treated with the carcinogen of interest, after which this plasmid DNA is transformed into yeast cells. In the FASAY assay, human cells are treated with the carcinogen of interest, after which plasmids containing *TP53* cDNAs generated by RT-PCR from these cells are transformed into yeast. In both of these assays, plasmid DNAs are recovered from the yeast clones exhibiting dysfunctional TP53 transcriptional regulation phenotypes and are sequenced for *TP53* mutations. Despite the low-throughput limitations, the resultant mutation spectrum is useful for analyzing the types of mutations induced by specific carcinogens.

Yeast genotoxicity tests based on transcriptional responses to DNA stressors

To maintain genome integrity, living organisms employ molecular mechanisms that can sense stress in the form of DNA damage and abnormal DNA replication arrest, both of which are caused by endogenous and exogenous factors. In prokaryotes, the SOS response pathway primarily detects DNA damage and, when triggered, activates DNA repair (Kreuzer 2013). Upon induction of the SOS response, "SOS genes" are rapidly transcribed. In the 1980s, two genotoxicity tests based on transcriptional activation of SOS genes in bacteria, the SOS chromotest (Quillardet et al. 1982) and the *umu* test (Oda et al. 1985), were developed. In eukaryotes, the DNA damage checkpoint pathway plays a crucial role in sensing emergent DNA stress. In this system, DNA lesions activate a cascade of protein kinases, leading to cell cycle arrest, apoptosis, and transcription of DNA repair-related genes for recovering damaged DNA and stalled DNA chain elongation (Sancar et al. 2004). Transcription of these genes is up-regulated when the DNA damage checkpoint pathway is activated by genotoxic agents (Elledge et al. 1993), indicating that the level of DNA damage from the genotoxicants can be estimated by measuring the transcript levels of these genes. Thus, several yeast-based genotoxicity reporter assays have been developed that use DNA stress-inducible promoters. These assays have been used to assess the genotoxicities of many compounds, including those in environmental samples and/or pharmaceuticals (Table 1). As described for the DNA alteration-based tests, dose-dependent elevation of the reporter in yeasts exposed to a test compound reflects the levels of compound-induced DNA stresses. The genotoxicity of the compound is often judged as positive after comparison with the levels of reporter induced by well-characterized DNA damaging agents and/or carcinogens. The yeast gene promoters and reporters used for these types of assays, the basic properties of the reporter assays, and additional novel yeast reporter assays are described briefly below.

Promoters

Microarray-based genome-wide expression studies have revealed that some of the 6000 *S. cerevisiae* genes are specifically induced by genotoxic stresses (e.g., Benton et al. 2006; Caba et al. 2005; Fry et al. 2006; Mizukami-Murata et al. 2010). Among this gene fraction, transcriptional regulation of the *RNR3* gene, which encodes a large subunit of ribonucleotide reductase, has been well-studied (Fu et al. 2008). Briefly, *RNR3* transcription is usually maintained at low levels by the Crt1p repressor binding to the promoter. However, upon DNA damage, the checkpoint kinase cascade is activated and Crt1p is phosphorylated by Dun1p kinase to release phosphorylated Crt1p and allow simultaneous binding of transcription activators. This process results in high levels of *RNR3* gene expression (Fig. 2). *RNR3* promoter-linked reporter assays have been developed and used in several studies testing genotoxicity (Boronat and Piña 2006; Ichikawa and Eki 2006; Jia and Xiao 2003; Jia et al. 2002; Ochi et al. 2011; Wei et al. 2013; Zhang et al. 2008, 2010, 2011). These reporter assays use various DNA stress-inducible promoters (Table 1), including *RAD54* (Afanassiev et al. 2000; Bartoš et al. 2006; Billinton et al. 1998; Boronat and Piña 2006; Bui et al. 2016; Cahill et al. 2004; Daniel et al. 2004; Hilscherová et al. 2010; Keenan et al. 2007; Knight et al. 2004, 2007; Lichtenberg-Fraté et al. 2003; Schmitt et al. 2005; Van Gompel et al. 2005; Walmsley et al. 1997; Walsh et al. 2005; Westerink et al. 2009; Zounková et al. 2007), *RAD51* (Liu et al. 2008), *RNR2* (Afanassiev et al. 2000; Ichikawa and Eki 2006; Lu et al. 2015), *HUG1* (Benton et al. 2007; Benton et al. 2008; Wei et al. 2013), and *PLM2* and *DIN7* (Bui et al. 2015). Notably, the "GreenScreen GC" *RAD54* promoter-linked green fluorescent protein (GFP) reporter assay has been used for assessing the genotoxicity of many chemicals and environmental samples (Table 1). Interestingly, previous studies found that there were different responses to genotoxicants between reporter assays using several different promoters (Bui et al. 2015; Jia et al. 2002). Therefore, the simultaneous use of yeast reporter strains with different types of DNA damage-responsive promoters may allow for more comprehensive screening of a wide variety of chemicals for their potential genotoxicities.

Reporters

Reporter systems based on enzymes and fluorescent proteins are most commonly used for yeast reporter assays (Table 1). The activities of enzymes such as β-galactosidase, β-glucuronidase, and luciferase can be easily quantified by colorimetry or luminescence, suggesting their usefulness as enzyme-based reporter assays for quantitative analyses. These intracellular enzymes must be carefully extracted from cells prior to applying assays. To address this limitation, Ochi and colleagues developed a yeast-based reporter assay using the

Table 1 Yeast-based genotoxicity tests using DNA damage-response reporters

Reporter	Reporter construct	Strain(s) (Ploidy)	Reagent(s) tested	Comments	Reference
RAD54 promoter					
GFP (Aequorea victoria)	Multi-copy plasmid	FF18734, FF18984 (haploid) WDHY669 (diploid)	MMS	DNA damage checkpoint-dependent induction of GFP with MMS	Walmsley et al. 1997
Yeast-enhanced GFP (yEGFP)	Multi-copy plasmid	FF18984	MMS, HU	Improved fluorescence detection by yEGFP	Billinton et al. 1998
			MMS and 18 other chemicals	96-well microplate format assay Comparable detection with the RNR2-yEGFP reporter	Afanassiev et al. 2000
			8 metals, 10 chemicals, and 34 industrial effluents	Cytotoxicity and genotoxicity assays using a portable instrument	Knight et al. 2004
			102 chemicals	96-well format genotoxicity and cytotoxicity test called the GreenScreen GC assay	Cahill et al. 2004
			38 effluents	Comparison of 7 ecotoxicity assays including the GreenScreen GC assay	Daniel et al. 2004
			2761 chemicals including 51 marketed pharmaceuticals	Usefulness of the GreenScreen GC assay plus the Ames test for screening genotoxic compounds under drug development	Van Gompel et al. 2005
			5 polyaromatic hydrocarbons and 20 aza-analogues	Sensitive or comparable detection by the GreenScreen GC assay compared with the SOS-chromotest	Bartoš et al. 2006
			305 chemicals	82% genotoxicity specificity by the GreenScreen GC assay	Knight et al. 2007
			5 anticancer compounds	Different genotoxicities in the GreenScreen GC assay compared with the SOS-chromotest	Zounková et al. 2007
			Raw and treated effluents	Ecotoxicity detection by the GreenScreen GC assay	Keenan et al. 2007
			River sediments	Ecotoxicity assays with the GreenScreen GC assay, the Microtox test and the SOS-chromotest	Hilscherová et al. 2010
		FYAK28/8-10B1 (haploid)	4-NQO and 2 mutagens, UV	Simultaneous cytotoxicity and genotoxicity detection	Lichtenberg-Fraté et al. 2003
		(FY1697-28C with pdr5Δsnq2Δyor1Δ)	Water samples	Evaluation of genotoxicity and cytotoxicity by dilution factor	Schmitt et al. 2005
		FF18984 and its 3 cell wall mutants Int022A (reporter-integrated strain)	11 chemicals for the mutant tests	Increased induction in the GreenScreen GC assay using the mutants	Walsh et al. 2005
			4 chemicals including AFB1 for the Int022A test	Detection of genotoxicities with 3 chemicals in the Int022A strain expressing human CYP3A4 or CYP1A2	Bui et al. 2016
		Y486 (FF18984)	MMS, AFB1, benzo[a]pyrene, N-nitrosodimethylamine	Genotoxicity-positive assay for AFB1 and benzo[a]pyrene with the strain expressing human CYP3A4, CYP2B6, or CYP2D6	Bui et al. 2016
β-galactosidase (β-gal)	Not described	SKAM4	254 chemicals	Luminescence-based sensitive assay called RadarScreen	Westerink et al. 2009
β-glucuronidase	Chromosomal integration	BY4743 (diploid)	MMS and 4 genotoxic chemicals	Highly sensitive detection using the RAD54-GUS reporter	Boronat and Piña 2006
RAD51 promoter					
Luciferase (firefly)	Single-copy plasmid	YPH499 (haploid)	MMS, 4-NQO and 26 chemicals	Dual-reporter assay (an integrated Renilla luciferase gene)	Liu et al. 2008
RNR2 promoter					
β-gal and LexA-Gal4p	Multi-copy sensor- and reporter plasmids	DF5, BY4741 (haploid)	MMS and 5 genotoxic chemicals	Sensitive detection of genotoxicants by the "indirect" reporter assay	Ichikawa and Eki 2006
yEGFP	Multi-copy plasmid	FF18984	MMS and 18 chemicals	Comparable detection as the RAD54-yEGFP reporter	Afanassiev et al. 2000
	Multi-copy plasmid	W303-1A (haploid)	MMS and 15 chemicals	Dual-reporter assay (a plasmid containing the DsRed-Express2 gene)	Lu et al. 2015

Table 1 (continued)

Reporter	Reporter construct	Strain(s) (Ploidy)	Reagent(s) tested	Comments	Reference
RNR3 promoter					
β-gal	Single-copy plasmid	DBY747 (haploid)	MMS and 10 chemicals, UV, γ-rays	Higher induction with MMS than with RNR2- and MAG1-lacZ reporters	Jia et al. 2002
	Chromosomal integration			Comparable induction in the integrated and plasmid reporters	
	Single-copy plasmid	DBY747 and its 9 DNA repair-deficient strains	MMS, EMS, 4-NQO, UV, γ-rays	Enhanced induction with alkylating agents in mag1Δ and with UV and 4-NQO in rad2Δ strains	Jia and Xiao 2003
		BY4741 and its cwp1Δ/cwp2Δ mutant	MMS, HU 4-NQO, phleomycin	Increased sensitivities in the RNR3- and DDI1-lacZ reporters using the deletion mutant	Zhang et al. 2008
		BY4741 and its cwp1Δ, cwp2Δ, snq2Δ, pdr5Δ strains	MMS, 4-NQO, phleomycin, chlorambucil, cisplatin	Enhanced induction of the RNR3-lacZ reporter in CWP and transporter genes deletion mutants	Zhang et al. 2010
		BY4741 and its deletion mutants including yap1Δ	MMS, 4-NQO, phleomycin, UV, γ-rays, 3 oxidants	Enhanced induction in the yap1Δ strain. Highest induction in the quintuplet mutant	Zhang et al. 2011
	Multi-copy plasmid	DF5	MMS and 4 genotoxic chemicals	Comparable induction in the RNR2- and RNR3-lacZ reporters	Ichikawa and Eki 2006
β-glucuronidase	Chromosomal integration	BY4743	MMS and 4 genotoxic chemicals	Fast detection by the RNR3-GUS reporter	Boronat and Piña 2006
Secretory luciferase (Cypridina noctiluca)	Multi-copy plasmid	DF5, BY4741 and its 12 DNA repair gene deletion mutants	MMS, EMS, phleomycin, HU, mitomycin C, and cisplatin	Highly enhanced induction with MMS, camptothecin, and mitomycin C in the assay with some deletion mutants	Ochi et al. 2011
GFPuv, stable and unstable luciferases (luc2 and luc2CP)	Multi-copy plasmid	BY4741 and its DNA repair or oxidative stress gene deletion mutants	MMS, HU, H_2O_2	Enhanced GFP induction in mag1Δ and mms2Δ strains. Poor responses of GFP and stable luciferase reporters for H_2O_2. Detection of oxidative stress by the TRX2 promoter-unstable luciferase reporter	Suzuki et al. 2017
GFP	Chromosomal integration	YB440 (diploid) (reporter-integrated strain expressing human CYP3A4)	AFB1, γ-rays	Genotoxicity detection of AFB1 by ectopic expression of human CYP3A4	Fasullo et al. 2017
HUG1 promoter					
GFP (S65T)	Chromosomal integration (HUG1 locus)	SPY810 (haploid)	MMS and 8 genotoxic chemicals, γ-rays	Sensitive genotoxin detection, comparable with other yeast-based reporters	Benton et al. 2007
		SPY810-derived mag1Δ and mre11Δ strains	MMS and 7 genotoxic chemicals, γ-rays	Enhanced GFP induction in the mag1Δ and mre11Δ strains	Benton et al. 2008
yEGFP	Chromosomal integration	BY4741-derived snq2Δ, pdr5Δ, cwp1Δ, cwp2Δ, yap1Δ, rad1Δ and mag1Δstrain	MMS, 4-NQO, phleomycin, chlorambucil, cisplatin, 3 oxidants	Sensitive detection of genotoxicants with the septuplet deletion mutant. Comparable detection with the RNR3-yEGFP reporter	Wei et al. 2013
PLM2, DIN7 promoters					
yEGFP	Multi-copy plasmid	Y486 (FF18984) (haploid)	MMS and 8 chemicals	More sensitive genotoxicant detection than the RAD54- and RNR2-GFP reporters	Bui et al. 2015
Gene promoters involved in DNA repair and DNA damage responses					
GFP (S65T)-fused proteins	Chromosomal integration (ORF-fused GFP gene)	BY4741-derived GFP clones	Nanomaterials (e.g., $nTiO_2$, carbon black) 16 chemicals including 11 genotoxicants	Cellular damage evaluation based on GFP expression profiles in the selected yeast reporter strains (>37 clones)	Lan et al. 2014
					Lan et al. 2016

GFP green fluorescent protein, MMS methyl methanesulfonate, HU hydroxyurea, yEGFP yeast-enhanced GFP, 4-NQO 4-nitroquinoline-1-oxide, AFB1 aflatoxin B1, EMS ethyl methanesulfonate

secretory luciferase gene from *Cypridina noctiluca*, whose activities can be readily quantified from the culture supernatant (Ochi et al. 2011). Fluorescent protein-based genotoxicity tests have also proved popular because they provide a convenient way of measuring the fluorometric signal using a microplate format. The codon-optimized yeast enhanced GFP (yEGFP), a GFP mutant derivative (Cormack et al. 1997), is commonly used in current reporter assays such as the GreenScreen GC assay (Billinton et al. 1998), while other GFP mutant derivatives such as GFPuv (Suzuki et al. 2017) and GFP(S65T) (Benton et al. 2007; Lan et al. 2014) are used in other assays. A dual-reporter assay using firefly and *Renilla* luciferases (Liu et al. 2008) or yEGFP and DsRed-Express2 (Lu et al. 2015) was developed to accurately determine the degree of mutagen-dependent reporter induction. Recently, Suzuki et al. examined genotoxic and oxidative stress responses in *S. cerevisiae* using the DNA damage-responsive *RNR3* promoter and the oxidative stress-responsive *TRX2* promoter, which drive stable or unstable luciferase reporter proteins, respectively. They found that stable luciferase proteins are suitable for reporting genotoxicity but not oxidative stress, while the opposite is true for unstable luciferase proteins (Suzuki et al. 2017).

Basic properties of the assay systems

Reporter induction in an assay is influenced by many factors, including the agent to be tested, its concentration, and its bioactivation status, and of course, the reporters, promoters,

and yeast strains themselves. With many of the assay systems, a minimum incubation period of 4–6 h in the presence of the genotoxic agent is required to obtain clearly detectable reporter induction, with the optimal time varying according to the aforementioned factors. The levels of reporter induction are frequently described as "fold induction," which is the ratio of the reporter activity in the presence and absence of the genotoxicant. The induction profile of the reporter depends largely on its concentration and the type of genotoxicant being tested. For example, in yeast cells treated with the alkylating agent methyl methanesulfonate (MMS), the reporter levels increase in a concentration-dependent manner, but they decrease markedly at high concentrations because of MMS cytotoxicity. It is also noteworthy that most procarcinogens and some antitumor drugs are poor inducers of reporter proteins. Detecting the genotoxicities of these compounds requires their activation by pre-treatment with an S9 extract or by exogenously expressed cytochrome P450s (CYP450s) in the yeast cells, or the use of sensitive reporter assays based on DNA repair-deficient strains (described later). A relatively wider range of mutagens can be detected with these assays than is possible with bacteria-based tests, as illustrated by the successful detection of several compounds that test negative in the Ames test. The limits of detection for MMS have been reported as approximately 3–90 μM in previous studies (Jarque et al. 2016), although the limit varies for each compound. However, the detectable concentrations of the compounds used in yeast-based assays are generally higher than those required for mammalian cell-based assays. This

Fig. 2 DNA stress-triggered induction of *RNR3* transcription via the DNA damage checkpoint pathway. Upon DNA damage or abnormally stalled DNA chain elongation, the Mec1p-Rad53p-Dun1p kinase cascade is activated and the Crt1p transcriptional repressor, which is bound to the X-element on the *RNR3* promoter, is phosphorylated. Phosphorylated Crt1p is released from the *RNR3* promoter and *RNR3* transcription is strongly induced with the assistance of Rdp3p, Wtm2p, and Hos2p transcription factors
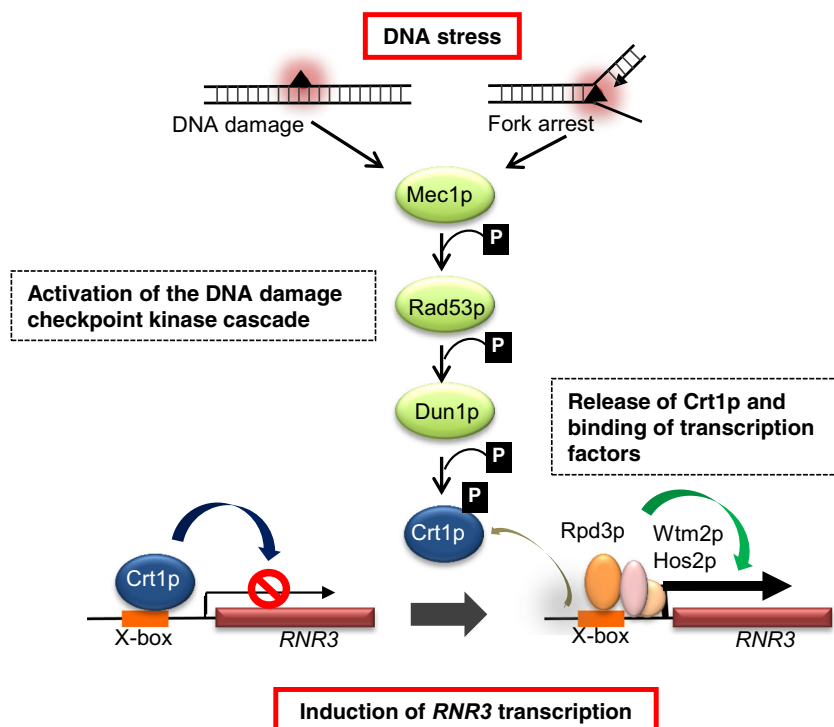
limitation is associated with the presence of cell walls in yeast, which is an issue that needs resolving in these assay types.

Novel yeast-based reporter assays

Ichikawa and Eki established a yeast-based reporter assay system that uses both an *RNR2* promoter-driven Gal4p-LexA sensor plasmid and a *lexA* operator-driven *E. coli lacZ*-reporter plasmid. This "indirect assay" system can be used to monitor low concentrations of genotoxic agents more efficiently than conventional reporter systems (Ichikawa and Eki 2006). Recently, toxicogenomics has shed light on the interactions occurring between genes and toxic substances via genome-wide analyses such as transcriptomics. Lan and colleagues used a library of yeast clones expressing GFP-fused DNA repair-related proteins to assess the potential genotoxicities of nanomaterials (Lan et al. 2014) and environmental pollutants (Lan et al. 2016). In these studies, the expression profiles of these GFP-fusion proteins in yeast clones were examined systematically in the presence of various types of genotoxicants, and the DNA damage types and relevant genotoxic mechanisms were predicted based on the characteristic expression patterns in the library.

Improvements to yeast-based genotoxicity tests

Yeast genotoxicity tests have been constantly updated to improve their sensitivities and their specific detection of potential genotoxicins in various samples. As described below, major improvements have been made by (1) improving cell permeability using cell wall synthesis and/or drug transporter mutants, (2) metabolic activation of procarcinogenic compounds with mammalian CYP450s, (3) sensitive detection using DNA repair-deficient mutants, and (4) using high-throughput assay formats.

Increased cell permeability

For broad and practical application, a genotoxicity test should be sufficiently sensitive to detect low levels of mutagenicity caused by chemicals and environmental pollutants. Because the yeast cell wall is a strong barrier against the influx of chemicals, the sensitivities of chemical-induced toxicities in yeast-based tests can be improved by increasing cell permeability. Improved sensitivities have been achieved in several DNA alteration-based tests by using yeast mutants with permeable membranes. One example is the D7ts1 strain, which is a derivative of D7 with the *sec53* mutation allele (Dimitrov et al. 2011; Morita et al. 1989; Pesheva et al. 2005, 2008; Staleva et al. 1996; Terziyska et al. 2000). Disruption of membrane transporter genes is another effective way of increasing

the sensitivity of genotoxicity screening in yeast-based reporter assays. For example, the transporter genes *PDR5*, *SNQ2*, and *YOR1* have been singly or multiply deleted (Lichtenberg-Fraté et al. 2003; Schmitt et al. 2005; Walsh et al. 2005; Wei et al. 2013; Zhang et al. 2010). An alternative approach is the disruption of cell wall-related genes to test reporter responses to genotoxicants, including mutations in the ergosterol biosynthesis gene, *ERG6*, and the cell wall mannoprotein genes, *CWP1* and *CWP2* (Ichikawa and Eki 2006; Walsh et al. 2005; Wei et al. 2013; Zhang et al. 2008, 2010). Enhanced induction of the reporters by genotoxicants in comparison with the parent strains has been detected in some of these multiple gene-deletion mutants (Walsh et al. 2005; Wei et al. 2013; Zhang et al. 2008, 2010).

Metabolic activation by CYP450 enzymes

In animals, many exogenous substrates such as procarcinogens and drugs are converted into reactive molecules by phase I oxidative enzymes and then "neutralized" by conjugation with phase II enzymes such as glutathione S-transferase. Lipophilic compounds are transformed into hydrophilic metabolites throughout this metabolic activation/detoxification process. The CYP450s, which comprise over 50 forms, are the most important phase I monooxygenases and are involved in the biotransformation of many compounds (Božina et al. 2009). Procarcinogens exhibit their genotoxic effects after being converted to DNA-reactive metabolites by CYP450-mediated enzymatic activation. Therefore, it is important to pre-treat test chemicals with mammalian metabolizing enzymes prior to bacterial genotoxicity tests because bacterial strains lack mammalian CYP450 systems. Malling et al. identified dimethylnitrosamine mutagenicity in bacterial tests after treatment with mammalian liver homogenates (Malling 1971), which led to the improved version of the Ames test using S9 rodent liver extracts (Ames et al. 1973). The S9 extracts that are used to bioactivate test compounds are often prepared from rodents treated with Aroclor 1254, to maximize the expression of liver enzymes, including CYP450s. Although yeast species have simple endogenous CYP450 systems, chemical bioactivation by their enzymes is insufficient and/or differs from that which occurs naturally in mammals. Indeed, Brusick and Mayer detected increased recombinogenic properties of alkyl-nitrosamines pre-treated with S9 liver extracts in an *S. cerevisiae*-based genotoxicity test (Brusick and Mayer 1973), and others have reported sensitive detection of genotoxic chemicals in yeast-based tests (Kirpnick et al. 2005; Liu et al. 2008; Pesheva et al. 2008; Stehrer-Schmid and Wolf 1995; Westerink et al. 2009). To overcome the need for whole animals and S9 extracts, yeast strains expressing a mammalian CYP450 system (co-expressing NADPH-CYP450 oxidoreductase and CYP450) were developed for assaying bioactivation-dependent toxicity, including genotoxicity (van

Leeuwen et al. 2012). To date, efficient detection of several procarcinogens, including aflatoxin B$_1$, using yeast strains expressing fly or human CYP450 systems has been reported in DNA alteration-based tests (Black et al. 1989, 1992; Del Carratore et al. 2000; Guo et al. 2005; Paladino et al. 1999; Saner et al. 1996; Sengstag et al. 1996; Sengstag and Würgler 1994) and in reporter assays (Bui et al. 2016; Fasullo et al. 2017; Walsh et al. 2005). Because many CYP450s bioactivate compounds in a substrate-specific manner and there are a wide range of such compounds, tests using a panel of yeast reporter strains each expressing a different mammalian CYP450 will be helpful for assessing procarcinogen genotoxicity in place of S9 extract-dependent tests.

Use of DNA repair-deficient mutants

Mutagens tend to cause specific types of DNA damage, and these lesions are repaired mainly by corresponding specific DNA-repair pathways. Although there are complementary pathways and there is cross talk between the different DNA-repair pathways, mutants defective in a specific repair pathway are generally hypersensitive to mutagens associating with just that pathway. For example, in yeast, breaks in double-stranded DNA that are caused by X-ray irradiation are repaired mainly by homologous recombination. Mutant strains that are defective in homologous recombination (for example, the $rad51\Delta$ strain) are highly sensitive to X-rays. Previous studies have shown that cell proliferation and viability in some yeast DNA repair mutants are markedly impaired by DNA damaging agents (Chang et al. 2002; Giaever et al. 2004; McKinney et al. 2013; Parsons et al. 2004; Wu et al. 2004). Therefore, cell proliferation assays and/or cytotoxicity tests using yeast DNA repair mutants can be used to detect genotoxic chemicals (Toussaint et al. 2006), to screen anticancer drug candidates (Simon et al. 2000), and to estimate the modes of action of anticancer drugs (Beljanski et al. 2004).

In several studies using yeast-based reporter assays, significantly higher levels of genotoxicant-induced reporters were detected in DNA repair-deficient cells than in wild-type cells (Benton et al. 2008; Jia and Xiao 2003; Ochi et al. 2011; Suzuki et al. 2017; Wei et al. 2013). This enhanced reporter induction likely results from the continuous activation of DNA-damage checkpoint kinases in response to unrepaired DNA lesions in the mutants. These studies clearly illustrate the potential of yeast DNA repair-deficient mutants for increasing the sensitivity of reporter-based genotoxicity tests. Furthermore, the type of DNA damage caused by a genotoxicant may be estimated from the induction profile of the reporter gene in a library of DNA repair pathway deletion mutants.

Zhang and colleagues also detected increased reporter levels after exposure of the *YAP1* deletion mutant to DNA-damaging agents. *YAP1* encodes an oxidative stress-responsive transcription factor, and the increased reporter levels were independent of oxidative damage (Zhang et al. 2011). Enhanced induction of Ty1 transposition by a mutagen was similarly observed in this mutant (Dimitrov et al. 2011). Although the molecular mechanism underlying these effects is unknown, the *YAP1* deletion mutant may be useful for increasing the sensitivity of yeast-based reporter assays.

Integration of high-throughput assays and microfluidic devices

High-throughput assays are essential for the short-term genotoxicity screening of large numbers of samples. Unlike DNA alteration-based assays, reporter-based assays are highly suited to high-throughput microplate screening because the colorimetric, luminometric, and fluorometric formats of these plates allow for easy quantitation. To date, 96-well microplate-format reporter assays have been established by several groups (Boronat and Piña 2006; Cahill et al. 2004; Lichtenberg-Fraté et al. 2003; Liu et al. 2008; Lu et al. 2015; Suzuki et al. 2017; Westerink et al. 2009), as well as a 384-well microplate format assay (Bui et al. 2015). Besides improving throughput, other technical developments are in progress that should increase the practicality of using yeast-based reporter tests. For example, the development of novel portable instruments and the integration of reporter assays into microfluidic devices or chips are important requirements for on-site genotoxicity tests. Accordingly, a genotoxicity sensing portable instrument has been developed based on the *RAD54* promoter-linked yEGFP reporter strain (Knight et al. 2004). García-Alonso and colleagues have also developed a microfluidic device for assaying genotoxicity using magnetized GFP reporter yeasts and have demonstrated its success in detecting MMS genotoxicity (García-Alonso et al. 2009, 2010, 2011). A chip device containing yeast-based reporter strains could be a powerful and convenient tool for on-site genotoxicity assays.

Conclusions and future perspectives

Genotoxicity tests are based on DNA alterations or on transcriptional responses to DNA damage. Since the 1970s, two types of *S. cerevisiae*-based genotoxicity tests have been developed and used as short-term genotoxicity screening tools for chemicals or polluted environmental samples. These tests have been used together with bacteria- and/or whole animal-based tests. The earlier types of yeast-based tests monitor a variety of DNA alterations, such as gene conversions and deletions, and are advantageous for genotoxicity assessment in comparison with the mutation-detecting Ames test. However, the usefulness of these tests is limited by their need for laborious plating work, and the resultant low throughput remains an unresolved issue. The more recently developed

genotoxicity tests detect genotoxic stress-induced transcriptional induction by using a yeast-based reporter system linked to a DNA damage-responsive promoter. These "yeast biosensors" share genotoxicity detection characteristics with other yeast-based tests and the Ames test and have high-throughput assay formats that can be easily up-scaled to screen large numbers of chemicals. To date, several mammalian cell-based reporter assays have been developed for genotoxicity testing (Hastwell et al. 2006; Hendriks et al. 2012; Rajakrishna et al. 2014; Westerink et al. 2010); however, yeast-based reporter tests offer advantages in terms of handling requirements and cost. Although the yeast-based tests have some remaining issues, including limited chemical permeation through the cell walls and insufficient endogenous bioactivation of procarcinogens, these issues can be partially addressed by the deletion of multiple chromosomal genes and the ectopic expression of mammalian CYP450 systems. Importantly, recent advances in CRISPR/Cas9-mediated genome editing allow rapid and precise deletion of multiple genes involved in cell wall synthesis, membrane transport, and DNA repair in yeast cells without laborious auxotrophic selection. Moreover, multiple chromosomal integration reporter strains containing reporter and mammalian CYP450 genes can be easily prepared by genome editing. The resultant "designer yeast biosensors" could greatly improve detection sensitivities and allow a wider variety of genotoxicants to be tested than current yeast reporter strains. It is expected that in the near future, cell array- or microfluidic device-based assays using these genetically enhanced yeast reporter strains will appear as next-generation, yeast-based genotoxicity tests.

Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

Ethical statement This article does not describe any studies with human participants or with animals that were performed by the author.

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