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RAD5a and REV3 function in two alternative pathways of DNA-damage tolerance in *Arabidopsis*

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ABSTRACT

DNA-damage tolerance (DDT) in yeast is composed of two parallel pathways and mediated by sequential ubiquitinations of PCNA. While monoubiquitination of PCNA promotes translesion synthesis (TLS) that is dependent on polymerase ζ consisted of a catalytic subunit Rev3 and a regulatory subunit Rev7, polyubiquitination of PCNA by Mms2–Ubc13–Rad5 promotes error-free lesion bypass. Inactivation of these two pathways results in a synergistic effect on DNA-damage responses; however, this two-branch DDT model has not been reported in any multicellular organisms. In order to examine whether *Arabidopsis thaliana* possesses a two-branch DDT system, we created *rad5a rev3* double mutant plant lines and compared them with the corresponding single mutants. *Arabidopsis rad5a* and *rev3* mutations are indeed synergistic with respect to root growth inhibition induced by replication-blocking lesions, suggesting that *AtRAD5a* and *AtREV3* are required for error-free and TLS branches of DDT, respectively. Unexpectedly this study reveals three modes of genetic interactions in response to different types of DNA damage, implying that plant *RAD5* and *REV3* are also involved in DNA damage responses independent of DDT. By comparing with yeast cells, it is apparent that plant TLS is a more frequently utilized means of lesion bypass than error-free DDT in plants.

1. Introduction

Cellular DNA of all organisms is constantly exposed to DNA-damaging agents from exogenous and endogenous sources. DNA alterations can lead to mutagenesis, genome rearrangements and cell death. To maintain genomic integrity, a variety of DNA repair mechanisms have evolved to protect cells from DNA damage [1]. Using the budding yeast *Saccharomyces cerevisiae* as a powerful model eukaryote, great advances have been made in the understanding of different DNA repair pathways. It has been well established that the *RAD3* nucleotide-excision repair pathway plays a critical role in the removal of DNA lesions induced by UV irradiation and bulky adducts [2]. The *RAD52* group genes are involved in homologous recombination (HR), which mainly repairs double-strand breaks (DSBs) induced by ionizing radiations, certain chemicals and the HO endonuclease [3]. In addition, other DNA repair pathways, including base-excision and mismatch repair, deal with specific lesions. Furthermore, instead of repairing the lesions encountered, DNA-damage tolerance (DDT), also known as DNA postreplication repair (PRR) in yeast, facilitates DNA synthesis in the presence of replication-blocking lesions [4]. This pathway is centrally controlled by genes belonging to the *RAD6* group [5].

Yeast PRR consists of two branches: an error-prone branch and an error-free branch. The error-prone tolerance mechanism is known as translesion synthesis (TLS) because it primarily utilizes low fidelity DNA polymerases to bypass lesions at the cost of increased mutagenesis [4]. These polymerases include Y-family polymerases such as Rev1 and Polη, as well as one B-family polymerase Polζ, which consists of a Rev3 catalytic subunit and a Rev7 regulatory subunit. In contrast, error-free PRR requires the ubiquitin (Ub)-conjugating enzyme (Ubc or E2)–Ubc E2 variant (Uev)–Ub ligase (E3) complex Ubc13–Mms2–Rad5 and bypasses replication-fork blocks perhaps via template switch [5,6]. It has been reported that these two subpathways are coordinated by sequential modifications of proliferating cell nuclear antigen (PCNA). In response to DNA damage, the Rad6–Rad18 ubiquitination complex monoubiquitinates PCNA at the Lys164 residue; the Ubc13–Mms2–Rad5 complex then further polyubiquitinates PCNA through Lys63-linked poly-Ub chains [7]. Thus, it is conceivable that monoubiquitinated PCNA promotes TLS, whereas polyubiquitinated PCNA promotes error-free PRR [8,9].

Genes involved in both branches of DDT have been isolated and characterized from the multicellular plant model organism *Arabidopsis thaliana*. In the TLS branch, genes encoding Rev3 [10], Rev1, Rev7 [11], Polκ [12], and Polη [13,14] have been reported.

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Inactivation of these genes causes variable sensitivity to UV as well as other DNA-damaging agents. Plant genes putatively involved in error-free DDT have also been identified, including two *UBC13* [15], four *UEV1* [16], and two *RAD5* [17] genes. *uev1d* [16] and *rad5a* [17] single mutants are sensitive to DNA-damaging agents. The observation that genes involved in yeast DDT are conserved in *Arabidopsis* [18] and mammals [9] suggests that the two-branch model of DDT is also conserved. However, to the best of our knowledge, this model has not been demonstrated in a multicellular organism. In budding yeast, mutations in TLS and error-free PRR have a synergistic effect, since *mms2/ubc13* or *rev1/rev3/rev7* single mutants are only moderately sensitive to DNA-damaging agents, but a double mutant defective in both branches becomes extremely sensitive [19–21]. In this study, we report that mutant plants defective in both *RAD5A* and *REV3* display a strongly enhanced sensitivity to certain DNA-damaging agents reminiscent of phenotypes observed in the yeast TLS and error-free PRR double mutant, hence providing evidence that the *RAD6* controlled DDT pathway is also conserved in plants.

2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana ecotype "Columbia" and their mutant derivatives were used in this study. The *rad5a* and *rev3* T-DNA insertion single mutant lines were obtained from the *Arabidopsis* Biological Resources Center. To generate the *rad5a rev3* double mutant, homozygous single mutant plants were obtained and crossed, and homozygous double mutants were identified in the F2 population by genomic PCR. The plants were grown in pots placed in a growth room (21 °C constant, 16/8 h day/night photoperiod with a daylight fluence rate of 140 μmoles/m^2/min).

2.2. Genomic DNA isolation and RT-PCR analysis

Total RNA was isolated using TRIzol (Invitrogen) from 9-day-old seedlings. Reverse transcript synthesis of the first-strand cDNA was performed with the ThermoScript RT-PCR kit (Invitrogen). Briefly, 2–4 μg of total RNA for each sample was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase. Experiments were performed using *AtRAD5A* and *AtREV3* gene-specific primer pairs. The same volume of each reaction was used for agarose gel electrophoresis.

2.3. Measurement of plant sensitivity to DNA-damaging agents

Sterilized seeds were placed in ½ Murashige and Skoog (MS) agar plates as previously described [16]. For the seed germination assay, the medium was supplemented with different concentrations of DNA-damaging agents and the plates were incubated in a growth chamber (continuous lighting at about 100 μmoles/m^2/min) for the period indicated before photography.

For the root growth assay, the plates were incubated vertically in the growth chamber. After 9 days, the root length of each seedling was measured using NIH ImageJ Software (version 1.42) and expressed as the percentage of the average length of untreated wild-type roots in the same experiments.

A modified root bending assay [22] was performed to assess plant sensitivity to UV light. Sterilized seeds were placed on the ½ MS agar plates and grown vertically for three days. The plates were then exposed to 1 kJ/m^2 of 254 nm UV light (FB-UVXL-1000 UV cross-linker, Fisher Scientific) every second day four hours after the beginning of the light period for a total of three (for the 9th-day measurement) or four (for the 12th-day measurement) exposures. Immediately after the first exposure, the plates were rotated 90° so that *de novo* root growth was redirected. The root length after "bending" was measured as described above.

2.4. Yeast strains and cell culture

S. cerevisiae wild type strain DBY747 (*MAT**a**, his3Δ1, leu2–3,112 ura3–52 trp1–289*) and its isogenic mutant strains WXY382 (*rev3Δ::LEU2*), WXY731 (*rad5Δ::hisG–URA3–hisG*) and WXY736 (*rev3Δ::LEU2 rad5Δ::hisG–URA3–hisG*) have been previously reported [23]. Yeast cells were grown in either the liquid YPD (1% yeast extract, 2% peptone, 2% dextrose) medium or on YPD + 2% agar plates at 30 °C.

2.5. Testing sensitivity of yeast cells to DNA-damaging agents

A serial dilution assay as previously described [24] was employed to determine yeast mutant sensitivity to MMS, 4-nitroquinoline 1-oxide (4NQO) and mitomycin C (MMC). For cisplatin-induced killing, overnight yeast cultures were used to inoculate fresh YPD. Cisplatin was added to the liquid culture at the given concentrations and samples were withdrawn at the indicated time. A serial dilution was made and spotted onto a YPD agar plate without the DNA-damaging chemical. For UV sensitivity, serially diluted yeast samples were spotted on YPD, exposed to 254 nm UV light in the UV crosslinker at given doses and incubated in the dark.

3. Results

3.1. Characterization of rad5a rev3 double mutants

Among available genetic tests capable of defining the yeast DDT pathway, the only convenient and critical criterion that can be applied to plants to date is the synergistic effects between mutations in error-free DDT and TLS. We selected *AtREV3* to represent the TLS branch since the *rev3* mutant is more sensitive to DNA-damaging agents than *rev1* or *rev7*, and the *rev3* mutation is epistatic to *rev1* and *rev7* [11]. In the error-free DDT branch, we found that while *ubc13* single mutants showed no noticeable phenotypes, the homozygous *ubc13a ubc13b* double mutants displayed multiple growth and developmental phenotypes in the absence of treatment with DNA-damaging agents (data not shown), suggesting that Ubc13 may be involved in cellular processes beyond DNA-damage response. This observation is understandable, as mammalian Ubc13 interacts with a panel of E3 ligases and is involved in several biological processes [25]. Since a Uev is absolutely required for Ubc13-mediated Lys63-linked polyubiquitination [26,27], we suspect that the four *AtUEV1* genes may also have distinct as well as overlapping functions, making them unsuitable for the proposed investigation. We turned our attention to plant homolog(s) of *RAD5*, the yeast cognate E3 ligase of Ubc13 required for error-free DDT [23,28], since E3s often provide substrate specificity.

After the identification of two *RAD5* homologs from *Arabidopsis* [18], our initial objective was to characterize *rad5a* and *rad5b* single and double mutants and then test the hypothesis that a two-branch DDT pathway exists in plants. While this investigation was in progress, Chen et al. reported that only *AtRAD5a* (*At5g22750*), but not *AtRAD5b* (*At5g43530*), is required for DNA-damage response. More importantly, the *Atrad5a Atrad5b* double mutant behaves the same as the *Atrad5a* single mutant [17], suggesting that *AtRAD5b* does not provide a backup function for *AtRAD5a*. This report prompted us to focus on the genetic interactions between TLS represented by *AtREV3* and the putative error-free bypass pathway represented by *AtRAD5a*. Our rationale was that if the two pathways corresponding to these two genes constitute two parallel branches