New *in vitro* dna polymerase activity and fidelity assay reveals age-dependent changes in *Arabidopsis Thaliana*

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Abstract

DNA polymerase is an enzyme that adds nucleotides to the growing DNA chain during replication and DNA repair. DNA polymerase activity and fidelity are important characteristics that reflect the ability of DNA polymerase to add nucleotides and then proofread newly synthesized DNA. We have developed a protocol allowing analysis of polymerase activity and fidelity using crude *Arabidopsis thaliana* plant extracts. It is based on the ability of DNA polymerases in the extract to elongate the fluorescently labelled primer annealed to a short complementary template. For analysis, fluorescently labelled products were separated on a denaturing polyacrylamide gel and visualized using a high performance blot imager. Analysis of tissue prepared from 5-, 12- and 21-day-old *Arabidopsis* plants showed an age-dependent decrease in polymerase activity, an increase in polymerase fidelity and a tendency to an increase in exo- (endo) nucleolytic activity.

Introduction

DNA polymerase fidelity reflects the ability of a polymerase to choose a correct nucleoside triphosphate (dNTP) and insert it in its proper position into a growing DNA strand. The role of DNA polymerases is crucial for efficient and precise genome replication as well as for DNA repair and recombination. The first polymerase discovered, *E. coli* Pol I, belongs to family A.[1] Besides its replicative function, *E. coli* Pol I contains two additional activities, a 3′-5′ and a 5′-3′ exonuclease. These activities are associated with proofreading activity because they can excise nucleotides misinserted by the polymerase. The eukaryotic member of this family, Pol γ, is a mitochondrial replicative enzyme.[1] Plants possess two *E. coli* Pol I homologs localized to plastids, *AtPolI-like* A and *AtPolI-like* B. These enzymes have high rates of expression in meristematic tissues and are induced by oxidative stress.[1] More recent work has showed that homologs of these polymerases in tobacco exhibit the replicative activity in chloroplasts and mitochondria.[2] Another polymerase belonging to family A is Pol θ that lacks proofreading activity and its role is not clear.[1] Pol θ has been shown to participate in the generation process of the antigenic variability of somatic hypermutation,[1] it is also thought to participate in DNA repair1 and possibly in translesion synthesis.[1] A possible homolog of Pol θ has been identified in rice.[1]

Most of the eukaryotic replicative polymerases belong to the B family of polymerases.[1] Among those are Pol δ and Pol ε, two main replicative polymerases, as well as Pol α, a primase. Pol δ and Pol ε contain an associated 3′-5′ exonuclease activity and are among the most faithful and processive enzymes.[1] The replication process is also dependent on dual activities of Pol α, a complex of a primase and a polymerase.[1] Pol δ and Pol ε are present in plants. Genetic evidence in *Arabidopsis* suggests that Pol ε is essential for replication.[1] Similarly, expression patterns of rice Pol δ are compatible with a role in the replication process.[1] One more polymerase belonging to family B is Pol ζ, an enzyme possibly involved in lesion bypass repair.[1] In *A. thaliana*, deletion of one of the Pol ζ subunits (*atrev3*) results in severe sensitivity to UVC,[1] suggesting that Pol ζ also plays a role in translesion repair in plants.

Another group of polymerases involved in filling in short gaps upon DNA repair belongs to family X. The most studied of these enzymes is Pol β that participates in base excision repair.[1] Other enzymes from this family are Pol λ and Pol μ that participate in the V(D)J recombination and strand break repair processes.[1] Pol λ is conserved in plants.[1] Plant Pol λ has 5′-deoxyribose phosphate lyase activity similar to mammalian and yeast homologs, suggesting that it may function in base excision repair.

The final group of polymerases belongs to family Y. These enzymes include Pol η, Pol κ and *AtREV1*. These enzymes have low affinity to undamaged DNA but can accommodate distorted DNA structures in their active site, resulting in the ability of these enzymes to bypass the DNA lesion polymerizing across the damaged DNA, a process that has been termed translesion synthesis.[1] For example, Pol η specializes in bypassing UV- induced lesions, cyclobutane pyrimidine dimmers, thus temporarily replacing replicative polymerases.

A. thaliana Pol η ortholog has been shown to complement Pol η deficiency in *S. cerevisiae* upon exposure to UVC, suggesting that this enzyme can participate in translesion synthesis in plants.[1] Moreover, *atrev1* plants are moderately UV-sensitive,[1] and thus it is possible that *AtREV1* is also involved in translesion synthesis.

The function of bacterial, yeast and mammalian polymerases have been extensively studied,[3,4] while plant polymerases largely remain in the shadow. DNA polymerase fidelity is analyzed using *in vitro* assays such as reporter gene assays,[5] pre-steady-state kinetic assays,[6] standard polymerase assays,[7] and gel assays.[8] All these techniques were applied to purified or partially purified DNA polymerases, and radioactive labels were used in most of them. In contrast, crude cell extracts are rarely used for the analysis of DNA polymerase activity.[9] A protocol allowing analysis of plant polymerase fidelity would be very useful for fundamental sciences and plant biotechnology. Here, we describe a modified and simplified protocol based on the classic work of Creighton and Goodman[8] for assessment of DNA polymerase activity and fidelity using crude plant extracts. In past, we used this protocol to show the role of polymerase fidelity/activity in changes in microsatellite stability in aged plants.[10] Here we present details of the development of this

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Key words: *Arabidopsis thaliana*, DNA polymerase fidelity and activity, plant age, a crude protein extract.

Acknowledgements: we would like to thank François Eudes and André Laroche from the Agriculture and Agri-Food Canada Lethbridge Research Centre for an unlimited access to core facilities and equipment in their laboratories. We are thankful to Valentina Titova for proofreading the manuscript. We would like to acknowledge NSERC Discovery and Alberta Agricultural Research Institute for providing funding to IK.

Contributions: AG, manuscript drafting, experiments performing; PM, manuscript writing and experiments performing; AB, experiments performing; IK, manuscript writing experimental work ideation and supervision.

Received for publication: 4 March 2011.
Revision received: 3 June 2011
Accepted for publication: 6 June 2011.

International Journal of Plant Biology 2011; 2:e7
doi:10.4081/pb.2011.e7

method and show that older plants exhibit a decrease in polymerase activity and fidelity. We also found that the exo-/endonuclease activity is lower in older plants. We further demonstrate that the use of purified protein extracts has no advantage over the use of crude protein extracts.

Materials and methods

Preparation of crude plant tissue extracts

For the experiment, *Arabidopsis thaliana* plants (ecotype Columbia-0) were used. Plants were grown at the temperature of 22°C during the day and 20°C during the night (12h day /12h night) period. For the pilot experiment, 12-day-old plants were used. DNA polymerase activity and fidelity were analyzed in 5-, 12- and 21-day-old *Arabidopsis thaliana* plants (ecotype Columbia). Leaf tissues were used for preparation of extracts. All extraction steps were carried out on ice. 10 g of tissue were homogenized using a pestle and a mortar in 100 mL pre-chilled homogenization buffer (100 mM sodium phosphate buffer, pH 7.4, containing 1% v/v protease inhibitor cocktail from Sigma, #P9599-5ML) to obtain a 10% extract. The extracts were filtered through four-fold layers of cheese cloth and centrifuged at 10,000 rpm for 15 min. The supernatant obtained was immediately used for assays. The assays were performed with crude extracts and extracts that were partially purified using the method described by Li *et al.* (2002).[11] The experiments were repeated three times.

Partial plant extract purification

All steps were performed in ice. Frozen plant tissues were grounded with liquid nitrogen and resuspended in 7 volumes (w/v) ice-cold homogenization buffer (25 mM Hepes-KOH, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 10% glycerol, 1 mM dithiothreitol, and protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany)). Homogenates were filtered through 45-µm filters and placed on a magnetic stirrer. 2M KCl was added slowly to the stirring homogenates to a final concentration of 450 mM. After 30 minutes of extraction, the homogenates were centrifugated at 40,000 g for 1 h to remove cellular and nuclear debris. The supernatants were transferred to glass beakers and solid $(NH_4)_2SO_4$ was added slowly to a final concentration of 70%. 10 µL of 1 M NaOH was added for each 1 g of $(NH_4)_2SO_4$ for supernatant neutralization. After 1 h incubation in ice, the precipitated proteins were collected by centrifugation at 20,000 g for 1 h. The supernatants were discarded, and the pellets were dissolved in the minimum volume (3 mL) of dialysis buffer (25 mM Hepes-KOH, pH 7.8, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 17% glycerol, 2 mM dithiothreitol) and dialyzed overnight against the same buffer (in 4 L) using 7,000 MWCO Slide-A-Lyser dialysis cassettes (Thermo Scientific). After dialysis, samples were split into aliquots, frozen in liquid nitrogen and kept at -80°C.

Determination of protein concentration

For the analysis of total protein concentration in crude plant tissue extracts, the Standard Bradford Assay with bovine serum albumin as a standard was applied.[12]

Preparation of the primer/template complex

In order to produce a primer/template complex for the assay, a fluorescein amidite (FAM)-labelled 15 nt primer (5' -6-FAM-TCCCAGTCACGACGT- 3', PAGE-purified) was annealed with a 30 nt template (5' -TCATC-GAGCATGATCACGTCGTGACTGGGA- 3', PAGE-purified). For that, all components were mixed by pipetting in the following order (on ice):

ΣV = 200 µL; Tris-HCl (1M, pH 8.0) = 10 µL; β-Mercaptoethanol (14.3M) = 0.5 µL; BSA (10 mg/mL, NEB) = 2 µL; Primer (100 µM) = 3 µL; Template (100 µM) = 3 µL; H_2O = 181.5 µL.

The reaction was incubated in a boiling water bath for 5 min and then cooled down slowly to room temperature (20-25°C). The primer/template complex was prepared in advance and stored at -20°C.

Another primer/template complex was prepared for the analysis of replication of G15 microsatellites. To do this, the FAM-labelled 15 nt primer (5' -6-FAM-TCCCAGTCACGACGT- 3', PAGE-purified) was annealed with a 30 nt template (5' -GGGGGGGGGGGGGGGACGTCGT-GACTGGGA- 3', PAGE-purified).

Polymerisation reaction

Arabidopsis thaliana crude extracts or partially purified extracts were prepared and split into three equal parts (10 µg of total protein in each). Therefore, crude extracts were used for the experiment immediately after preparation. In fact, we recommend that the preparation of plant extracts and reaction setup is done by two people simultaneously. Each part was mixed with the primer/template complex, the necessary 2 mM dNTP and reaction buffer (10x Y+/Tango buffer, Fermentas; 1x Y+/Tango buffer consists of 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA): ΣV = 25 µL, dNTP (2 mM) = 2.5 µL, Template/Primer complex = 2.5 µL, Reaction Buffer = 2.5 µL, Plant extract = 10 µg of total protein, H2O = to 25 µL.

The reaction was carried out at 37°C for 15 min in a PCR machine, quenched with 50 µL loading buffer (95% formamide, 50 mM EDTA, and 0.05% bromphenol blue), heated to 95°C for 3 min and cooled on ice for 2 min (it is possible to store the quenched reactions at -20°C for at least 4 weeks). The amount of plant extract for the reaction was calculated based on the Bradford assay data. The Klenow enzyme in NEB buffer 2 with the same primer/template complex but without the plant extract was used as a positive control. Each experiment was performed three times.

Denaturing polyacrylamide gel electrophoresis

After quenching, 15 µL of the sample/loading buffer mixture was loaded on a 20 % polyacrylamide gel (20×20×0.075 cm) containing 8 M Urea. Also, 0.5 µL of the 5 bp 6-FAM-labelled primer was mixed with 2.5 µL of loading buffer and loaded on the polyacrylamide gel (served as a molecular weight marker). Electrophoresis was carried out in 1x TBE buffer for about 5 h at 500 V (Bromphenol Blue should be approximately 2 cm from the end of the gel).

Gel scanning

Gels were scanned using Typhoon 9410 at an excitation wavelength of 488 nm using a 520 band pass 40 emission filter, the photomultiplier tube voltage of 685 V, at a resolution of 100 µm.

Calculation of polymerase activity, polymerase fidelity and exonuclease activity

Polymerase activity was calculated by relating the intensity of the expected band (16 nt in case of incorporation of dGTP and 17 nt in case of incorporation of dGTP and dATP) to the intensity of all bands in the individual lane. Polymerase fidelity was calculated by relating the intensity of bands appearing due to the misincorporation of dNTPs (17 nt band in case of dGTP incorporation and 18 nt band in case of dGTP+dATP) to the intensity of bands appearing due to the correct incorporation (16 nt in case of incorporation of dGTP and 17 nt in case of incorporation of dGTP and dATP). For the analysis of exonuclease activity, the intensity of the 14 nt bands was related to the total intensity of bands in the correspondent lanes.

Image analysis

Images of scanned gels were analyzed using ImageQuant 5.2 software (Molecular Dynamics). For the adjustment of total values of the object's outline, the Local Median background correction method was applied.

Statistical analysis

The experiments were repeated three times.

In each case, the assays were also run in triplicates, and the mean values ± SD were calculated. The statistical significance of the results was confirmed by performing a Student's t-test. Statistical analyses were performed using Microcal Origin 6.0.

Results and Discussion

The assay for the analysis of polymerase activity and fidelity is based on the ability of polymerases in crude protein extract to extend a FAM-labelled 15 nt primer by base-pairing with a 30 nt complimentary template (Figure 1). First, we performed several pilot experiments to establish the polymerase fidelity assay. The experiments revealed that the level of DNA polymerase activity is sufficiently high if the reaction is incubated for 10 or 20 min (Figure 1S). Thus, all further reactions were performed for 15 min. In a different experiment, we found that if extracts were used in ≥30 min after preparation, we did not detect any polymerase activity. Thus, from this point onward, the experiments were performed with freshly prepared or immediately frozen extracts.

In other preliminary experiments, we tested several reaction buffers from various vendors as well as self-made buffers. Among them, Fermentas buffer Y+/Tango showed the best results (Figure 1S). For pilot experiments, we used tissues from 12-day-old plants.

Figure 2 shows a typical gel after scanning and brightness/contrast adjustments. Two regions of gel can be selected for the analysis: one region above a 15 nt band and the second one – below. The region of 15 nt corresponds to the primer size. All bands above 15 nt fragments are polymerization products. All bands below 15 nt fragments are degradation products indicating the level of exo- (endo) nuclease activities. Depending on the starting material used, some unidentified fluorescent spots can be present on the gel. They usually do not interfere with polymerisation products and are very likely to come from plant tissues. As the first nucleoside of a template is cytidine, it was expected that dGTP will be ligated to the FAM-labelled 15 nt primer. Thus, it was expected that the reaction with dGTP would have a 16 nt product (Figure 2). The lane with dGTP had the most intense band as compared to other single NTPs; the band consisted of 15 nt and 16 nt (Figure 2). The appearance of the 16 nt product in the reaction with other dNTPS would suggest misincorporations, possibly due to polymerase mistakes (Figure 2). Such misincorporations were most noticeable in the reaction with dATP (Figure 2). This lane contained two extra bands, of 17 nt and 18 nt; the appearance of the band of 17 nt would be explained by the misincorporation of dATP at the position of 16th nucleotide and the correct incorporation of dATP at the position of 17th nucleotide. The appearance of an 18 nt band would be due to the misincorporation of dATP at the position of 18th nucleotide. The appearance of the 18 nt product would be due to two misincorporations instead of guanine at the position of 16th nucleotide and instead of thymine at the position of 18th nucleotide.

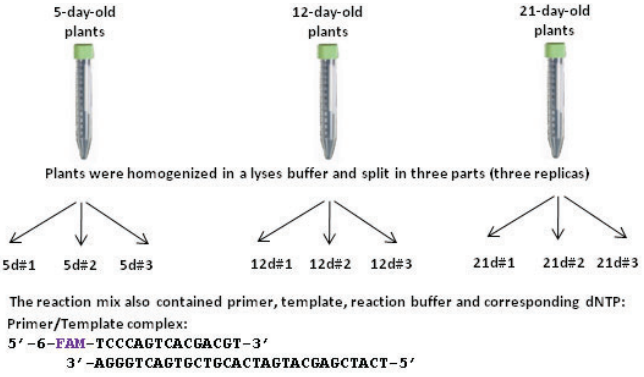

Figure 1. A general outline of the experiment. *A. thaliana* plants were grown for 5, 12 and 21 days. Whole plants without roots were harvested, and tissues from each age group were distributed for three independent repeats. The experiments were repeated three times (three biological repeats).

The correct incorporation of two nucleotides was observed in the pair of dGTP and dATP (17 nt in lane 5, Figure 2). Products larger than 17 nt in size, like in the case of dGTP + dATP (lane 5), will be formed due to misincorporated nucleotides, thus indicating the level of polymerase fidelity. Samples with all dNTPs (abbreviated as dNTPs) served as positive controls (the 30 nt product was produced; Figure 2). The appearance of a 17 nt band in lane 5 and a 16 nt band in lane 1 (dGTP) and lane 6 (dNTPs) allows analysis of polymerase activity. Samples without dNTPs (abbreviated as "-", lane 7) served as negative controls and showed the nuclease activity primarily (Figure 2). At the same time, samples abbreviated as dNTPs + 10 mM EDTA (Figure 2, lane 8) exhibited no signs of enzymatic activity – the presence of bands smaller or larger in size than 15 nt fragments. We used the Klenow polymerase activity (samples abbreviated as K, Figure 2, lane 9) as a positive control. In the case of Klenow, two main bands were produced, presumably of 29 and 30 nt. Analysis of the intensity of bands smaller in size than 15 nt will also allow analysis of exo- (endo) nuclease activity. Note that the intensity of bands smaller than 15 nt in size was equal in lanes 1-7 (Figure 2).

Next, we analyzed polymerase fidelity and exo- (endo) nucleolytic activities in plants of different age. We used tissues of 5-, 12- and 21-day-old *Arabidopsis thaliana* plants (ecotype Columbia-0) for the experiment. Comprehensive information as to the expression of various polymerases in plants of different age is scarce. Information on differences in polymerase fidelity in plants also does not exist. Only several papers suggest that the expression of plant polymerases is typically higher in germinating seeds and meristematic tissues.[13] We hypothesized that plants of different age would have a different level of polymerase fidelity and nuclease activity.

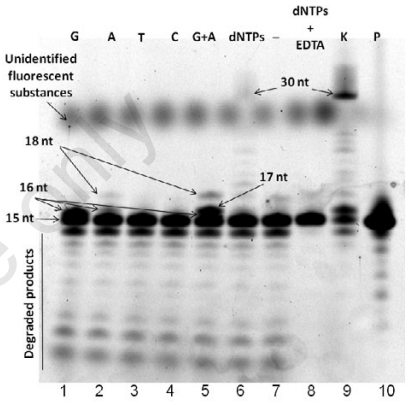

Figure 2. A typical gel image after scanning and brightness/contrast adjustments. Twelve-day-old plants were used for the analysis. Crude protein extract was prepared. "G" – dGTP, "A" – dATP, "T" – dTTP, "C" – dCTP, "G+A" – dGTP+dATP, "-" – no dNTPs were added, "K" – the Klenow fragment reaction products, "P" – the 15 nt 6-FAM labelled primer only. 15, 16, 17, 18 and 30 nt fragments are pointed by arrows. Unidentified fluorescent spots are marked. The lane with the primer was overloaded, and thus, it shows several faint bands indicating shorter products, intermediates of primer synthesis.

We used the same FAM-labelled primer and tested the incorporation of each of labelled dNTPs. Moreover, we also performed the analysis of incorporation of two dNTPs (dGTP+dATP), incorporation of all dNTPs, incorporation of all dNTPs in the presence of EDTA inhibitor (dNTPs + EDTA) as well as of a negative control in which dNTPs were absent. All reactions were performed with either crude or purified extracts. We noted that crude extracts had much higher activity as compared to purified extracts (Figures 2S-9S). Despite the fact that reactions with crude extracts

resulted in a high exonucleolytic activity, we used crude extracts because of its higher sensitivity.

The incorporation of dGTP and dATP, as reflected by the intensity of the band of 17 nt, was found to be lower in older plants (Figure 3 A). To analyze the polymerase activity, we related the intensity of the 17 nt band to the total intensity of all bands in the correspondent lane. This calculation showed that polymerase activity indeed decreased with age (Figure 3 A,B). The measurement of the intensity of the 18 nt band that appears due to the misincorporation of a single nucleotide at the position 18 of the template also showed a decrease with age; a lower intensity of 18 nt bands indicates less frequent misincorporations and thus higher fidelity (Figure 3A,C). Finally, the analysis of intensity of the 14 nt band showed a slight age-dependent increase. To analyze exonuclease activity, the intensity of the 14 nt bands was related to the total intensity of bands in the correspondent lanes. The exonuclease activity was found to increase with age, although the data were significant in the 21-day-old group only (Figure 3 A,D).

The analysis of the dGTP incorporation did not allow us to precisely calculate the polymerase activity since the 15 and 16 nt fragments overlapped substantially. Our attempts to separate these two bands were not successful. The analysis of the intensity of 17 nt fragments that was a result of a misincorporation at the position 17 of the template showed an age-dependent decrease in; thus, the polymerase fidelity in the reaction with dGTP increased with plant age (Figure 4 A,B). The analysis of 14 nt fragments showed an equal exonuclease activity (Figure 4 A,C).

The analysis of the reaction with dATP showed a lower intensity of 18 nt fragments in older plants; thus, the polymerase fidelity in the reaction with dATP increased with plant age (Figure 5 A,B). The analysis of 14 nt fragments showed an equal exonuclease activity (Figure 5 A,C).

The similar analysis was performed for the incorporation of dCTP, dTTP and all dNTPs. Whereas the reactions performed with crude extracts showed a trend similar to dGTP + dATP, dGTP and dATP, the reactions with purified extracts did not allow the analysis of polymerase fidelity as the polymerase reaction was completely abolished (Figures 5S-7S). The reaction with no dNTPs added also showed the difference between crude and purified extracts; there was a substantial decrease in exonucleolytic activity in reactions with purified extracts (Figure 8S). The reaction with dNTPs + 10 mM EDTA performed with purified extracts showed complete inhibition of enzymatic reactions (Figure 9S). As it can be seen, the intensity of all bands is similar among all samples prepared from either young or old tissues Figure 9S, part A). It can be thus concluded that variations in band intensities observed in previous gels are in fact due to age-dependent differences.

There is no information on dependence between polymerase fidelity and age in plants; therefore our findings are truly novel. In fact, the most recent review on DNA polymerases published by Garcia-Diaz and Bebenek and specifically devoted to plant polymerases does not contain any information on polymerase fidelity in plants.[1] The amount of information

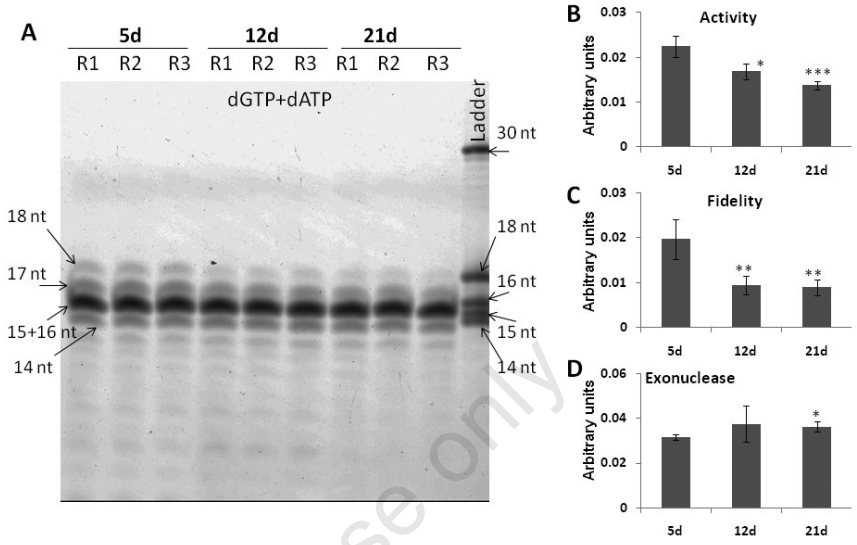

Figure 3. Analysis of dGTP +dATP incorporation. (A) Crude extracts from 5, 12 and 21 day old Arabidopsis thaliana plants (ecotype Columbia) were incubated in triplicates (R1-R3, R4-R6, R7-R9) with 1 mM dGTP and 1 mM dATP. The 14, 15, 16, 17 and 18 nt fragments are indicated by arrows. Ladder – shows the mix of labelled primers. (B) The average intensity (in arbitrary units ± SD, as calculated from 3 bands per each gel, with 3 independent replicates) of the 17 nt band represent polymerase activity. Asterisks show significant differences in 12 and 21 day old plants as compared to 5 day old plants, where one asterisk is P<0.05 and three asterisks are P<0.001. (C) The average polymerase fidelity (in arbitrary units ± SD) as calculated from measuring intensity of 18 nt fragments. Asterisks show significant differences in 12 and 21 day old plants as compared to 5 day old plants (P<0.01). (D) The average exonucleolytic activity (in arbitrary units ± SD) as calculated from measuring intensity of 14 nt fragments. Asterisks show significant difference in 21 day old plants as compared to 5 day old plants (P<0.05).

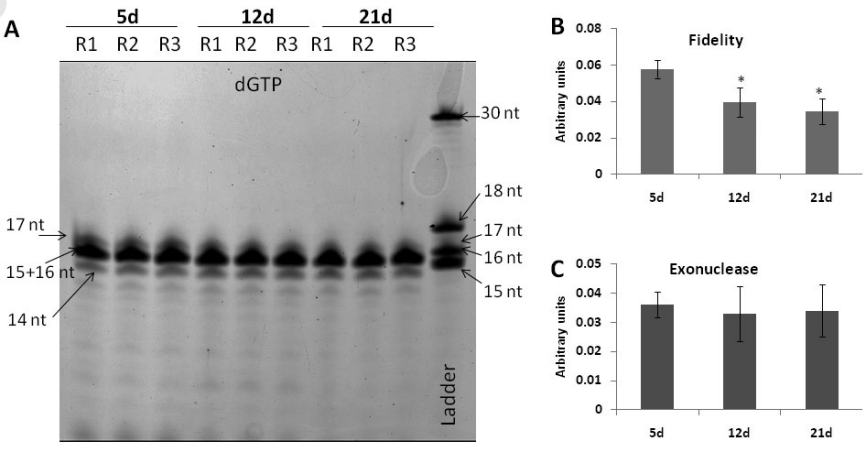

Figure 4. Analysis of dGTP incorporation. (A) Crude extracts from 5, 12 and 21 day old Arabidopsis thaliana plants (ecotype Columbia) were incubated in triplicates (R1-R3, R4-R6, R7-R9) with 2 mM dGTP. The 14, 15, 16, 17 and 18 nt fragments are indicated by arrows. Ladder – shows the mix of labelled primers. (B) The average polymerase fidelity (in arbitrary units ± SD) as calculated from measuring intensity of 17 nt fragments. Asterisks show significant differences in 12 and 21 day old plants as compared to 5 day old plants (P<0.01). (C) The average exonucleolytic activity (in arbitrary units ± SD) as calculated from measuring intensity of 14 nt fragments.

on the impact of age on polymerase activity in plants is also scarce. Bottomley suggests lower polymerase activity in older pea plants,[14] whereas Grilli et al. and Reuzeau and Cavalie show lower activity of RNA poly (A) polymerase in aging seeds.[15,16] Both authors demonstrated that RNA polymerase activity directly correlates with level of seed imbibition suggesting that moisture content plays a positive role in affecting the polymerase activity.[15,16] The polymerase activity depends on many factors, one of which could be expression of genes coding for various polymerases. Although many papers report various polymerase activities in different plants and different tissues,[16] we found very few papers dealing with the analysis of polymerase expression in plants of different age. For example, a Y-family polymerase was shown to be expressed at a high level in endoreduplicating *Arabidopsis* tissues.[17]

The ability to correct replication errors is one of the functions of replicative polymerases. The main replicative polymerases such as Pol δ, ε or γ (*AtPolI-like A* and *AtPolI-like B* in plants) are highly accurate, with mutation rates from 10^{-6} to 10^{-5}. Polymerases belonging to the X family, Pol β and Pol λ, have lower fidelity, with average error rates between 10^{-3} and 10^{-4}. Finally, translesion polymerases belonging to the Y family have the highest error rates of 10^{-1}-10^{-3}.[1] Since we found an age-dependent increase in polymerase fidelity, it can be hypothesized that the frequency of replication-related nucleotide misincorporations in older plants may be lower than in younger plants. In contrast, several papers suggest that mutation rates increase in aging plants.[18] Chwedorzewska *et al.* reported increased variations in AFLPs and other genetic markers in naturally aged rye (*Secale cereale*) seeds.[19] *Zea mays* plantlets,[20] *Triticum* plantlets[21] and *Crepis* plantlets[22] produced from older seeds had higher levels of chromosome rearrangements and various other mutations. All the above mentioned studies provide only indirect evidence for higher mutation frequencies in older plants. In our previous studies, we found an increase in the expression of several bypass and translesion polymerases, therefore, it can be hypothesized that the activity of these error-prone polymerases contributes to increasing mutation rates in older plants.[10] Alternatively, it is possible that higher rates of mutations in older plants are associated with lower ability of either mismatch repair proteins or excision repair proteins to deal with existing mismatched and damaged nucleotides.

While analyzing polymerase activity using crude extracts, we noticed high nuclease activity. In contrast, the nuclease activity decreased dramatically if purified proteins were used. Whereas the data obtained from crude extracts

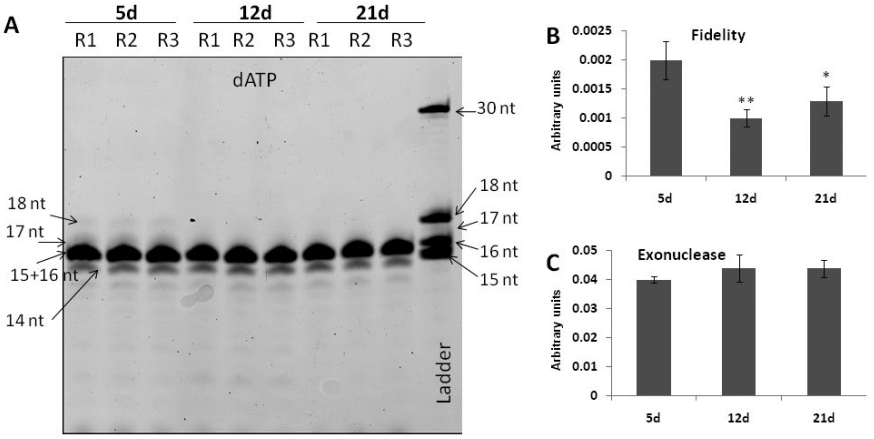

Figure 5. Analysis of dATP incorporation. (A) Crude extracts from 5, 12 and 21 day old *Arabidopsis thaliana* plants (ecotype Columbia) were incubated in triplicates (R1-R3, R4-R6, R7-R9) with 2 mM dATP. The 14, 15, 16, 17 and 18 nt fragments are indicated by arrows. Ladder – shows the mix of labelled primers. (B) The average polymerase fidelity (in arbitrary units ± SD) as calculated from measuring intensity of 17 nt fragments. Asterisks show significant differences in 12 and 21 day old plants as compared to 5 day old plants (P<0.01). (C) The average exonucleolytic activity (in arbitrary units ± SD) as calculated from measuring intensity of 14 nt fragments.

suggest an age-dependent decrease in nuclease activity, the data obtained from purified extracts show the similar exonuclease activity and even a slight tendency to an age-dependent increase in the nucleolytic activity. As it can be seen from Figure 10S, the exonucleolytic activity detected in the reaction with dCTP and dATP increased with age; it is possible that higher nuclease activity can be present if polymerases are not able to incorporate dNTP into the growing nucleotide chain.

The data on nuclease activity in plants are somewhat contradictory as no systematic attempt to analyse different tissues and different plant ages has ever been made. Analysis of the fate of exogenous DNA bombarded into young and old leaves of creeping bentgrass showed much higher degradation of DNA molecules in younger leaves.[23] The authors attributed this effect to a higher level of nucleases in younger leaves. In fact, the authors showed that pre-treatment with nuclease inhibitors prevented DNA degradation. In contrast, Aleksandrushkina *et al*. showed that older pea leaves had higher nuclease activity.[23] Using gel electrophoresis to detect exo-(endo) nucleolytic degradation of DNA molecules delivered into cells and protoplasts of carrot plants, Schaefer *et al.* showed higher exogenous DNA degradation in older cells.[23]

Conclusions

Here we presented a new *in vitro* assay for assessing the activity and fidelity of plant DNA polymerases. Our assay allows to compare polymerase activity, polymerase fidelity and exonucleolytic activity of plants of different ages. It remains to be shown whether the assay would also detect differences among various plant tissues or in different genetic backgrounds, such as weak DNA polymerase mutants. The assay may also allow to test the efficiency of various buffers in preventing exonucleolytic activity. For better efficiency of the assay, we recommend to analyze the polymerase activity using crude protein extracts that are either freshly prepared or immediately frozen.

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