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BRIEF COMMUNICATION

Activity of DNA polymerase λ in spikelets of rice and maize

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Abstract

Involvement of DNA polymerase (pol) enzymes in meiotic DNA repair has not been clearly understood in plants. DNA polymerase λ is involved in short patch DNA synthesis and base excision repair in both plants and animals. The presence and activity of the pol λ enzyme was studied in a protein isolated from spikelets during flower development stages of rice (*Oryza sativa* L.) and maize (*Zea mays* L.). Western blot analysis showed a 2- to 2.5-fold higher accumulation of pol λ in spikelets than in shoots. Assays of pol and in-gel activity showed the dideoxynucleotide triphosphate sensitive pol λ enzyme in spikelets of both the plants. An enhanced presence of the enzyme and its high activity suggests an active role of pol λ in meiotic recombination during microspore development.

Additional key words: DNA repair, in-gel activity assay, meiosis, *Oryza sativa*, *Zea mays*.

Polymerases of DNA (pols) are extremely important enzymes required for replication as well as repair of genetic material of every organism. Several pol enzymes have been discovered and distributed into seven different families: A, B, C, D, X, Y, and RT (Rothwell and Waksman 2005). The members of X family pols are single polypeptides, dideoxynucleotide triphosphate (ddNTP) sensitive enzymes, having a nearly similar structure (Yamtich and Sweasy 2010) and efficiently filling single nucleotide gaps (Sobol *et al.* 1996). They are mostly involved in short patch DNA repair like base excision repair (Sarkar *et al.* 2004) and translesion DNA synthesis (Amoroso *et al.* 2011). DNA polymerase λ (pol λ) is the only X family pol present in plants (Uchiyama *et al.* 2009). DNA polymerase β (pol β; 39 kDa in mammals) is the smallest member of the X family, shares many common features with pol λ except a breast cancer type 1 susceptibility protein C terminus (BRCT) domain. This domain is important for protein-protein interaction (Zhang *et al.* 1998), and it is present at the N terminal end of pol λ but absent in pol β.

Recombination in meiosis involves physical interaction between two homologous chromosomes, and it is completed only after repair of double strand breaks. Meiotic recombination requires involvement of multiple enzymes. Many reports suggested that the members of X family pols are involved in meiosis (Plug *et al.* 1997, Garcia-Diaz *et al.* 2000, Kidane *et al.* 2010), but their role in meiosis of plants have not been proved beyond doubt. A high expression of pol λ has been reported in testes of mouse (Garcia-Diaz *et al.* 2000), which suggested its probable role in meiosis. It has also been found that pol β overexpression stimulates homologous recombination (Canitrot *et al.* 2004). But in plants, there are only a few reports which show involvement of pol in meiotic recombination. Previously, we have purified a ddNTP sensitive pol from shoot tips of rice (Sanathkumar *et* al. 1996), and its involvement in base excision repair was shown (Sarkar *et al.* 2004). A dideoxynucleotide triphosphate sensitive 67 kDa DNA polymerase purified from *Oryza sativa* ssp *indica* cv. IR8 was reported as pol β. But later on, after discovery of pol λ in mice (Garcia-Diaz *et al.* 2000) and in *O. sativa* ssp. *japonica* (Uchiyama *et al.* 2004), we concluded that the enzyme purified from rice cv. IR 8 was pol λ. Pol β has not been found in plants (Uchiyama *et al.* 2009). A

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Abbreviation: β-ME - β-mercaptoethanol; ddNTP - dideoxynucleotide triphosphate; PCNA - proliferating cell nuclear antigen; pol - DNA polymerase; TCA - trichloroacetic acid.

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high expression of pol λ was found in rice during seed germination and after salt stress (Sihi *et al.* 2015). The objective of this work was to study activity and expression of pol λ in spikelets of two agriculturally important plants, rice and maize, to determine its possible role during microspore development.

The seeds of *O. sativa* L. ssp *indica* cv. IR 8 were collected from the Chinsurah Rice Research Institute, West Bengal, India, and the seeds of *Zea mays* L. hybrid K-51 were collected from a local market. Spikelets were collected from plants grown in separate plots in the field. Young panicles (still inside the flag leaf) were collected from the field and the total buffer soluble proteins were isolated from 5 g of tissue. Proteins were also isolated from shoots of four-d-old rice and maize seedlings. Isolation of proteins was done according to Sarkar *et al.* (2004) in a cold room at 4 °C. Proteins were isolated in buffer A [50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 250 mM sucrose, 1 mM β-mercaptoethanol (β-ME), 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and after centrifugation at 10 000 *g* for 10 min, the supernatant was collected. Proteins were precipitated with ammonium sulphate to a 70 % saturation. The pellet was resuspended in 1 cm^3 of buffer A and dialysed against 500 volumes of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM β-ME, 0.1 mM EDTA, 20 % (v/v) glycerol and 1 mM phenylmethylsulfonyl fluoride]. Further purification was done through *DEAE-Sephacel* (*Sigma-Aldrich*, St. Louis, USA) column chromatography as we did earlier (Sarkar *et al.* 2004, Sihi *et al.* 2015).

In vitro pol assay of the purified protein was done according to Sarkar et al. (2004) with a slight modification. For assay of the rice enzyme in 50 mm^3 of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 10 mM dithiothreitol, 10 % (v/v) glycerol, 50 µM each of dATP, dGTP, and dTTP, and 1.85 × 10^5 Bq of α-^{32}P-dCTP, 20 µg cm^{-3} activated calf thymus DNA was taken. For assay of the maize protein 100 mM KCl plus 0.5 mM $MnCl_2$ were used. The reactions was carried out at 37 °C for 30 min and they were terminated by adding ice cold 10 % (m/v) trichloroacetic acid (TCA) containing 100 mM sodium pyrophosphate and incubated 30 min on ice. The precipitate was collected on a GF/C filter (*Whatman*) and washed with 2 % TCA twice using a *Millipore* (*Merck*, USA) vacuum filtration device. Radioactivity was measured by a liquid scintillation counter (*Tri-carb-2800TR*, *Perkin Elmer*, USA). Enzyme activity was measured by incorporation of α-^{32}P-dCMP in an activated calf thymus DNA, which functions as template primer for the pol enzyme.

Immunodetection of pol λ was done by Western blot analysis as we have done earlier (Sarkar *et al.* 2004, Sihi *et al.* 2015). Twenty µg of 70 % (m/v) ammonium sulphate purified and dialysed proteins was separated using 10 % (m/v) SDS-PAGE with standard protein molecular mass markers. Then, the proteins were electro-transferred to a polyvinylidine fluoride (*Amersham Biosciences,* USA) membrane using a mini vertical electrophoresis system (*Amersham Biosciences*) according to the manufacturer's instruction. After processing, the blot was hybridized with affinity purified polyclonal anti-sera (purified the IgG fraction) developed against rat pol β and then further processed with an alkaline phosphatase conjugated goat anti IgG secondary antibody solution. Primary antibody recognised bands were developed in nitroblue tetrazolium chloride (225 µg cm^{-3}) + 5-bromo-4-chloro-3-indolyl phosphate (175 µg cm^{-3}) solution.

Activity of pol λ was also detected by in-gel analysis following the methods of Karawya *et al.* (1983) and Blank *et al.* (1983). The resolving gel was made with 125 µg cm^{-3} of activated DNA. Protein samples were prepared by mixing 10 mm^3 of a sample buffer [4 % (m/v) special quality SDS, 2 % (v/v) β ME, 5 µg cm^{-3} bovine serum albumin, 15 % (v/v) glycerol] with 25 mm^3 (2 µg) of the *DEAE-Sephacel* purified protein. The electrophoresis gels were immersed in an SDS wash buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM β-ME, and 20 % (v/v) isopropanol and then in a protein renaturation buffer (50 mM Tris-HCl, pH 7.5, 5 mM β-ME, and 0.1 mM EDTA). Each gel was then incubated in a reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 10 mM dithiotreitol, 10 % (v/v) glycerol, 8 µM each of dATP, dGTP, and dTTP, and 3.7 × 10^5 Bq cm^{-3} of α-^{32}P-dCTP] for 30 min. The gels were thoroughly washed in 6 % (m/v) TCA with 1 % (m/v) sodium pyrophosphate, dried in a gel drier (*Hoeffer*, USA), and exposed to a *Kodak X-Omat* film for 24 h.

Flowering in grasses passes through several stages as initiation of inflorescence, flower structure development, heading or panicle extrusion, and anthesis or flower opening (Yoshida and Nagato 2011). Proteins were isolated from young spikelets in the stage just before heading and anthesis. Detection of pol activity was done in *DEAE-Sephacel* purified protein by incorporation of α-^{32}P-dCTP in pol assay in the presence and absence of the inhibitor ddCTP. Proteins isolated from spikelets of both rice plants and maize plants were sensitive to the inhibitor ddCTP and pol λ activity was higher in rice than in maize (Fig. 1*A*,*B*).

The polyclonal antibody developed against rat pol β detects pol λ of rice (Sarkar *et al.* 2004, Sihi *et al.* 2015) and *Arabidopsis* (Roy *et al.* 2011). The same antibody was used to detect the presence and level of expression of pol λ in rice and maize. A high expression of pol λ was found in the ammonium sulphate purified protein of both the studied species (Fig. 2*A*,*B*). The expression of pol λ was 2- to 2.5-fold higher in spikelets than in shoots of the 4-d-old plants. Protein molecular mass of pol λ in spikelts was 67 kDa in rice and 61 kDa in maize.

In situ gel analysis is a powerful tool to detect the catalytic subunit of the pol enzyme. Proteins were separated in standard sodium dodecyl sulphate poly-acrylamide gel electrophoresis, the polymerase enzyme was renaturated and assay was done to detect the catalytic subunit of the enzyme in the gel. The pol λ is a single polypeptide enzyme and activity of the enzyme was detected in-gel in both the plants. Protein purified by *DEAE-Sephacel* was loaded onto the gel and pol activity was detected (Fig 3*A*,*B*). The Klenow enzyme was used as positive control. Almost a 3.5-fold higher activity of the enzyme was found in rice spikelets than in rice seedling shoots and a slightly higher activity in maize spikelets than in seedling shoots.

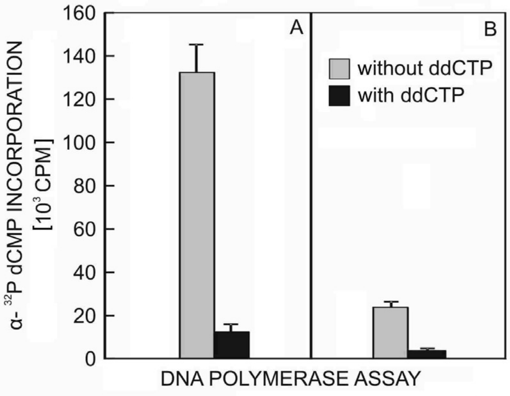

Fig. 1. Activity of dideoxycytidine triphosphate (ddCTP) sensitive DNA polymerase λ (pol λ) in rice (*A*) and maize (*B*) spikelets. Activity of pol λ was measured by *in vitro* DNA polymerase assay with equal amounts of the *DEAE-Sephacel* purified protein, and it was done without and with the inhibitor ddCTP. Incorporation of α-^{32}P dCMP was measured by a liquid scintillation counter (CPM - count per minute).

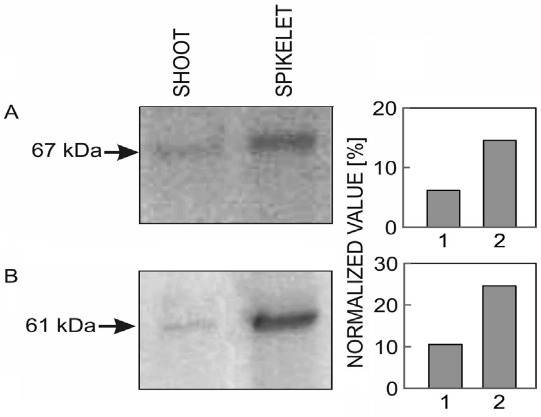

Fig. 2. Expression of DNA polymerase λ in seedling shoots and spikelets of rice (*A*) and maize (*B*). Western blot analysis of the ammonium sulphate purified protein isolated from 4-d-old shoots (1) and spikelets (2) with the primary antibody, and normalized values of densitometric scanning reports of the respective lanes.

Efficient repair of double strand breaks during meiosis are important for viable microspore and megaspore formation in plants (Caryl *et al.* 2003). Repair DNA synthesis is essential part of meiotic recombination repair (Jasin and Rothstein 2013). Though it is well understood that DNA synthesis occurs during meiotic recombination, involvement of specific pol enzymes has not been properly characterised. Various reports suggested that during meiosis pols as pol δ (Maloisel *et al.* 2008), pol ζ (Sharma *et al.* 2012), pol η and pol κ (Sebesta *et al.* 2013), are involved apart from pol λ (Garcia-Diaz *et al.* 2000) and pol β (Plug *et al.* 1997, Kidane *et al.* 2010). In rice pol λ was shown to be present in meiotic tissue (Uchiyama *et al.* 2004). In a recent review, Luo *et al.* (2014) mentioned a list of 28 genes with known function in meiosis of rice, but the involvement of pol has not been included.

We have collected spikelets from the proper stage of inflorescence when they undergo anther development and meiosis (Chen *et al.* 2005) and prepared the *DEAE-Sephacel* purified protein. As a very little incorporation of radioisotope occurs in DNA polymerase assay with the ammonium sulphate purified protein, pol activity was measured with the *DEAE-Sephacel* purified protein. High enzyme activities were observed in both the cases and the enzyme was inhibited by the pol λ inhibitor ddCTP. The enzyme assay showed a high activity of pol λ, which indicates its active role in spikelets. An elevated level of pol λ activity is found in imbibed seeds of rice, which suggests its role in DNA repair after imbibition (Sihi *et al.* 2015). The enzyme assay result directed us to study expression of pol λ by immunoblot analysis.

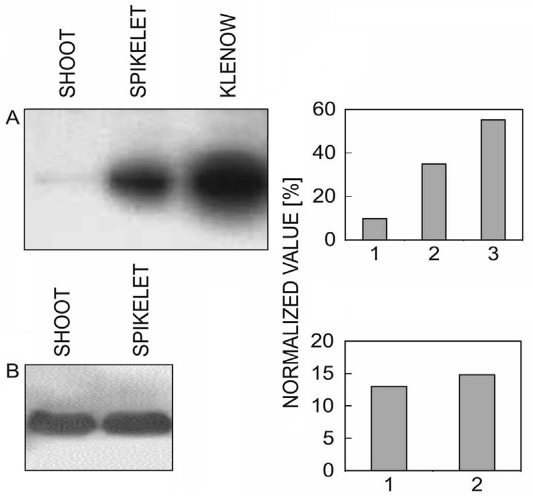

Fig. 3. In gel activity of DNA polymerase λ in shoots (1) and spikelets (2) of rice (*A*) and maize (*B*). In-gel activity assay of the *DEAE-Sephacel* purified protein. One U of Klenow enzyme was used in *A* as positive control. Normalized values of densitometric scanning reports of the respective lanes.

Western blot analysis shows a 2 to 2.5 fold enhanced accumulation of the pol λ enzyme in spikelets than in seedlings. The enzyme from maize is smaller than the enzyme from rice (Sarkar *et al.* 2004) and more close to the enzyme from *Arabidopsis* (Roy *et al.* 2011). We tested our results with in-gel activity assay and it showed a high pol λ activity of the *DEAE-Sephacel* purified protein from spikelets of both the plants but higher in rice than in maize. The pol λ in maize probably require other protein factors for its maximum activity in spikelets. Nevertheless, the presence of the active ddNTP sensitive enzyme suggests its requirement in spikelets of both rice and maize.

DNA polymerase λ is known to be involved in repairing DNA *via* non-homologous end joining in *Arabidopsis* (Furukawa *et al.* 2015). During meiotic recombination, SPOrulation (Spo 11) creates double strand breaks generating 3′ overhangs, which ultimately lead to D loop formation. Subsequent DNA synthesis extends 3' and produce Holliday junction (Liu *et al.* 2013). It was indicated that proliferating cell nuclear antigen (PCNA) is required in that reaction (Wang *et al.* 2004). In plants reports show that pol λ interacts with PCNA (Amoroso *et al.* 2011) and so it might play a role in recombination repair with the help of other proteins like PCNA.

Our results show the presence of a highly active ddNTP sensitive pol in the spikelets. Being the sole member of the X family of pols in plants (Uchiyama *et al.* 2009), pol λ probably plays an important role in meiotic recombination repair in rice and maize.

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