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The OsGEN-L protein from *Oryza sativa* possesses Holliday junction resolvase activity as well as 5′-flap endonuclease activity

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OsGEN-L has a 5′-flap endonuclease activity and plays an essential role in rice microspore development. The Class 4 RAD2/XPG family nucleases, including OsGEN-L, were recently found to have resolving activity for the Holliday junction (HJ), the intermediate of DNA strand recombination. In this study, we performed a detailed characterization of OsGEN-L, as a structure-specific endonuclease. Highly purified OsGEN-L was prepared as the full-length protein for *in vitro* endonuclease assays using various structured DNAs, and the 5′-flap endonuclease activity, which is stimulated in a PCNA-dependent manner, was demonstrated. In addition, the *in vitro* HJ resolving activity of OsGEN-L represents the first such activity originating from plant cells. OsGEN-L cleaved HJ at symmetrically related sites of the branch point. However, the two branched strands seemed to be cleaved individually, and not cooperatively, by each OsGEN-L monomer protein. The substrate specificity suggests that OsGEN-L functions in multiple processes of DNA metabolism in rice cells.

Keywords: DNA repair/flap endonuclease/Holliday junction/PCNA.

Abbreviations: HJ, holliday junction; PCNA, proliferating cell nuclear antigen; FEN, flap endonuclease; XPG, xeroderma pigmentosum group G.

Genome integrity and stability are critical for all living organisms. However, there are many endogenous (replication error, oxidative damage and hydrolytic damage) and exogenous (mutagens, UV and ionizing radiation) factors in the environment that damage DNA (*1*). A variety of nucleases are employed in different pathways of DNA metabolism to maintain the integrity of the genetic information on the genome. The RAD2/XPG family is well known as an important substrate-specific nuclease family involved in DNA replication, repair and recombination (*2*, *3*).

The RAD2/XPG family members are divided into four classes, based on the molecular size and the locations of two conserved domains, the XPG-N and XPG-I domains. Class I consists of the XPG-like proteins. XPG (Xeroderma pigmentosum group G) and RAD2 have ssDNA-specific endonuclease activity and 5′–3′ exonuclease activity for both ssDNA and dsDNA, and are involved in nucleotide excision repair (NER) (*4–6*). Human XPG is important in cancer healing (*7*, *8*). Class II is the flap endonuclease-1 (FEN-1), which possesses 5′-flap endonuclease (FEN), gap endonuclease (GEN) and 5′–3′ dsDNA exonuclease activities (*9–11*). The multiple nuclease activities of FEN-1 work in numerous DNA metabolic pathways, including Okazaki fragment maturation, stalled fork repair, telomere maintenance, base excision repair and apoptotic DNA fragmentation. Class III represents Exonuclease 1 (EXO1), which shares a remarkably conserved nuclease domain with FEN-1, and displays both 5′–3′ exonuclease activity and FEN activity (*12–14*). Exo1 functions in the mismatch repair (MMR) and double-strand break repair (DSBR) reactions involved in DNA replication, repair, recombination and telomere integrity. EXO1 is also crucial for cell DNA damage checkpoint activation.

In addition to these three classes, Class IV was proposed most recently when a new enzyme, designated as OsSEND-1, was discovered from *Oryza sativa* (*15*). The homologous sequence of OsSEND-1 is conserved only in plants. OsSEND-1 possesses single-strand DNA endonuclease activity, and the expression of its gene is induced by UV and DNA-damaging agents, such as methyl methanesulfonate (MMS) and H_2O_2. Therefore, OsSEND-1 is proposed to have some important role in the plant DNA repair system. DmGEN, which was discovered as an XPG-like endonuclease from a search for an XPG homolog in the *Drosophila* expressed sequence tag (EST) database, is another member of the Class IV enzymes. Class V was originally proposed from a phylogenetic analysis using the amino acid sequences (*16*), but DmGEN is now recognized as belonging to Class IV (*17*). In addition to the wide conservation of DmGEN homologs in animals and plants, DmGEN has an endonuclease activity for various flap structures, including replication fork models (*17*). A cDNA encoding a sequence homologous to that of DmGEN was cloned from an *O. sativa* library, and the gene product, named OsGEN-L (OsGEN-like), was characterized (*18*).

The OsGEN-L protein has FEN activity and plays an essential role in rice microspore development (*18*).

The molecular mechanism of homologous recombination has been studied extensively, and the proteins involved in the process were identified in *Escherichia coli* (*19*, *20*). After the discovery of Rad51, the functional counterpart of bacterial RecA in eukaryotes, the identification of the enzyme that resolves the Holliday junction (HJ), the recombinational intermediate, as the counterpart of the bacterial RuvC proteins, still remained as a target (*21–24*). Recently, a report describing the eukaryotic HJ resolvases was published, in which the DmGEN homologs in human (GEN1) and yeast (Yen1) were identified by MS spectrometry of an extensive activity fractionation from a HeLa cell extract and an assay of the cell extracts from a *Saccharomyces cerevisiae* TAP fusion library, respectively (*25*). Furthermore, the detailed molecular mechanisms of the HJ resolution reaction by GEN1 were analysed, and revealed that their properties are reminiscent of those of RuvC, the classical *E. coli* HJ resolvase (*26*). The GEN1/Yen1 proteins in class IV of the RAD2/XPG family are now recognized as eukaryotic HJ resolvases (*27*, *28*).

We have been interested in the HJ resolvases in Archaea. We identified a protein with HJ resolvase properties from *Pyrococcus furiosus*, a hyperthermophilic archaeon (*29*) and analysed its biochemical and structural properties in detail (*30–32*). The protein, designated as Hjc, is conserved only in Archaea, and no homolog has been found in Eukaryota and Bacteria. When we surmised that GEN1 might be a eukaryotic HJ resolvase, we initiated an investigation of whether the OsGEN-L protein has HJ resolvase activity. We now present the detailed biochemical properties of OsGEN-L, determined using the highly purified protein. In addition to the proliferating cell nuclear antigen (PCNA)-dependent FEN activity, OsGEN-L displayed HJ resolution activity.

Materials and Methods

Sequence analysis

The sequence of OsGEN-L (accession no. AB158320) was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov). The disorder tendency of the OsGEN-L protein structure was predicted by using the IUPred software (*33*).

Preparation of cDNA library from the developing seeds of O. sativa

Poly(A)$^+$ RNA was prepared from developing rice seeds (10–14 days after flowering) as described earlier (*34*). The synthesis of single-strand cDNA and the following construction of the cDNA library used λZAPII vector (Agilent Technology) were performed according to the packaged manual.

Plasmid construction for the expression of the gene encoding OsGEN-L

We modified pET21a (Novagen) by inserting sequences encoding a His-tag, TEV protease recognition, and NcoI recognition between the NdeI and BamHI sites, and designated the resultant plasmid as pET21a-HTC. The coding region of *OsGEN-L* was amplified from a rice cDNA library by PCR with the primer set of OsGen5 (5′-ggccatgggggtgggggcagcttctg-3′) and OsGen3 (5′-ggaagcttttagtcgaacagcaggcgacgacggac-3′), containing NcoI and HindIII restriction sites, respectively (underlined). After digestions by NcoI and HindIII, the *OsGEN-L* fragment was inserted into the corresponding site of pET21a-HTD. In the plasmid, the *OsGEN-L* fragment is connected in-frame to the His-tag gene under the T7 promoter. The sequence of the constructed plasmid, pOsGENL, was determined using a CEQ2000XL analyser (Beckman Coulter), to confirm the nucleotide sequence of the *OsGEN-L* gene on the expression plasmid.

Overexpression and purification

The pOsGEN-L plasmid was introduced into *E. coli* BL21-CodonPlus (DE3) -RIL cells (Agilent Technology). The transformed *E. coli* cells were incubated in 4 l of Luria-Bertani medium (MERCK), containing 34 μg/ml chloramphenicol and 50 μg/ml ampicillin, at 37°C. When the absorbance of the culture at 600 nm reached 0.6, IPTG was added to 1 mM to induce the *OsGEN-L* gene expression and the incubation was continued for 16 h at 16°C. The cells were collected, washed with a buffer containing 10 mM Tris–HCl, pH 8.0 and 0.1 M NaCl, and suspended in 50 ml of buffer A (50 mM potassium phosphate, pH 8.0, 0.5 M NaCl, 10 mM imidazole, and 1 mM PMSF) for sonication. After 10 min of ultrasonication (Astrason Ultrasonic Processor), the lysate was centrifuged at 24,000 × *g* (Beckman Coulter Avanti HP-25) for 10 min. The supernatant was then loaded onto a Ni-NTA Agarose column (QIAGEN). The bound proteins were eluted by buffer A containing 250 mM imidazole. The eluted proteins were dialysed against buffer B (20 mM Tris–HCl, pH 7.5, 10% glycerol, 0.4% Nonidet P-40, 1 mM DTT and 0.3 M NaCl). The dialysed solution was then loaded onto a 1 ml HiTrap Heparin HP column (GE Healthcare), equilibrated with buffer B, and the bound proteins were eluted with a 10 ml linear gradient of 0.3–1 M NaCl, using an AKTA purifier (GE Healthcare). The fraction containing the OsGEN-L protein was dialysed against buffer C (40 mM Tris–HCl, pH 8.8, 10% glycerol, 0.04% Nonidet P-40, 1 mM DTT and 0.2 M NaCl), and was loaded on a 1 ml HiTrap Q HP column (GE Healthcare) equilibrated with buffer C. The bound proteins were eluted by a linear gradient of NaCl from 0.2 to 1 M. All purification processes were performed at 4°C. The purified OsGEN-L was dialysed against a buffer containing 40 mM HEPES–KOH, pH 7.4, 10% glycerol, 0.04% Nonidet P-40, 1 mM DTT and 0.2 M NaCl, and was stored at −80°C.

Gel filtration analysis

The purified OsGEN-L was subjected to gel filtration on a Superdex 200 3.2/30 column (GE Healthcare), with a mobile phase of solution, containing 50 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA and 0.3 M NaCl. The chromatography was performed with a SMART system (GE Healthcare). The loading volume was 40 μl. The molecular masses of the proteins were estimated from the elution profiles of the standard marker proteins (Bio-Rad), including thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000) and myoglobin (17,000).

Preparation of OsPCNA

We modified pET21d by inserting sequences encoding a His-tag, Tobacco Etch Virus (TEV) protease recognition, and NdeI recognition between the NcoI and BamHI sites, and designated the resultant plasmid as pET21d-HTD. The nucleotide sequence of the gene for OsPCNA was retrieved from the database (accession no AK071591), and the PCNA-coding region was amplified from a rice cDNA library (as described above) by PCR with the primer set of OsPCNA-5 (5′-gggcatatgctggagctgaggcttgtgc-3′) and OsPCNA-3 (5′- ggggcggccgcttacgacttcatttcctcatcttcttcaatc-3′), containing NdeI and NotI restriction sites, respectively (underlined). After digestions by NdeI and NotI, the OsPCNA fragment was inserted into the corresponding site of pET21d-HTD. In the plasmid, the OsPCNA fragment is connected in-frame to the His-tag gene under the T7 promoter. The sequence of the constructed plasmid, pOsPCNA, was analysed with a sequencing analyzer, CEQ2000XL (Beckman Coulter), to confirm the nucleotide sequence of the OsPCNA gene on the expression plasmid. The recombinant *E. coli* cells bearing pOsPCNA were subjected to the same cultivation, cell lysis and the affinity chromatography using a Ni-NTA Agarose column as in the case of OsGEN-L. The fraction was dialysed against buffer D (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol). The dialysate was then loaded onto a 1 ml HiTrap Heparin HP column (GE Healthcare) equilibrated with buffer D, and the flow through fraction was loaded onto a

Table I. Oligonucleotides.

Name	Nucleotide sequence (5′–3′)
49N	AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT
49N2	AGCTACCATGCCTGCACGAATTCGTATCAGCGTAATCATGGTCATAGCT
49NA	AGCTACCATGCCTGCACGAATTAAAAAAAACGTAATCATGGTCATAGCT
49R	AGCTATGACCATGATTACGAATTGCTTAATTCGTGCAGGCATGGTAGCT
prim19	AGCTATGACCATGATTACG
prim20	AGCTATGACCATGATTACGA
prim25	AGCTATGACCATGATTACGAATTGC
prim26	AGCTATGACCATGATTACGAATTGCT
d27	AGCTATGACCATGATTACGAATTGCTT
d27+A	AGCTATGACCATGATTACGAATTGCTTA
d27+T	AGCTATGACCATGATTACGAATTGCTTT
F5-d42	GATGTCAAGCAGTCCTAAGGAATTCGTGCAGGCATGGTAGCT
F3-d47	AGCTATGACCATGATTACGAATTGCTTGGAATCCTGACGAACTGTAG
20(F5-d42)	GATGTCAAGCAGTCCTAAGG
21(F5-d42)	GATGTCAAGCAGTCCTAAGGA
d20	CTACAGTTCGTCAGGATTCC
d22	AATTCGTGCAGGCATGGTAGCT
d28	CTACAGTTCGTCAGGATTCCAAGCAATT
d26(49N2)	TACGAATTCGTGCAGGCATGGTAGCT
d27(49N2)	ATACGAATTCGTGCAGGCATGGTAGCT
d28(49N2)	GATACGAATTCGTGCAGGCATGGTAGCT
d29(49N2)	TGATACGAATTCGTGCAGGCATGGTAGCT
d30	AATTGCTTAATTCGTGCAGGCATGGTAGCT
HJ-1	CCTGCAGGATGGTAGGACGGCCTCGCAATCGGCTTCGACCGAGCACGCGAGATGTCAACGATCGAATTGC
HJ-2	GCAATTCGATCGTTGACATCTCGCGTGCTCGGTCAATCGGCAGATGCGGAGTGAAGTTCCAACGTTCGGC
HJ-3	GCCGAACGTTGGAACTTCACTCCGCATCTGCCGATTCTGGCTGTGGCGTGTTTCTGGTGGTTCCTAGGTC
HJ-4	GACCTAGGAACCACCAGAAACACGCCACAGCCAGGAAGCCGATTGCGAGGCCGTCCTACCATCCTGCAGG
HJm-1	CCTGCAGGATGGTAGGACGGCCTCGCAATCCCGATTGACCGAGCACGCGAGATGTCAACGATCGAATTGC
HJm-4	GACCTAGGAACCACCAGAAACACGCCACTCGGTCAATCGGGATTGCGAGGCCGTCCTACCATCCTGCAGG
HJm-2(59)	GCAATTCGATCGTTGACATCTCGCGTGCTCGGTCAATCGGCAGATGCGGAGTGAAGTTC
HJm-3(59)	GAACTTCACTCCGCATCTGCCGATTGACCGAGTGGCGTGTTTCTGGTGGTTCCTAGGTC

1 ml HiTrap Q HP column (GE Healthcare) equilibrated with the same buffer. The bound proteins were eluted by a linear gradient of NaCl from 0 to 0.5 M. All purification processes were performed at 4°C, and the purified OsPCNA was stored at −80°C.

Preparation of DNA substrates

All DNA substrates were formed by the oligonucleotides shown in Table I. All of the substrates used were prepared as: single strand (49N), 5′-overhang (49N, d27), 3′-overhang (49N, d22), gap (49N, prim19, d22), nick (49N, d27, d22), duplex (49N, 49R), bubble (49R, 49NA), splayed arms (F5-d42, 49N), 3′-flap (49N, F3-d47, d22), 5′-flap (F5-d42, 49N, d27), gapped flap [F5-42, 49N, and prim20 (or prim25, or prim26)], double-flap [F5-d42, 49N, F3-d47 (or d27+A, d27+T)], fork-gap [49N2, F3-d47, d28, and d22 (or d26 (49N2), d27 (49N2), d28 (49N2), d29 (49N2)], fork (49N, F3-d47, d22, d20) and Holliday Junction (HJ-1, HJ-2, HJ-3, HJ-4). One of the oligonucleotides (the first oligonucleotide in each bracket above) in each substrate was labelled at the 5′-end with [γ-^{32}P]ATP (PerkinElmer) by T4 polynucleotide kinase (New England Biolabs). The oligonucleotides were annealed by boiling in a solution containing 40 mM Tris–acetate, pH 7.8, and 0.5 mM Mg–acetate for 5 min followed by gradual cooling by a PCR machine (1 cycle: 98°C for 5 min, from 90°C to 40°C for 2 h slope).

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis was performed on a BIACORE J with a CM5 chip (GE Healthcare). The His-tagged OsPCNA was immobilized on the CM5 chip as a ligand by amine coupling, according to the manufacturer's protocol (GE Healthcare). The OsGEN-L solution was loaded onto the chip at 25°C, using 10 mM HEPES–NaOH, pH 7.4, 0.15 M NaCl and 3 mM EDTA, at a flow rate of 30 µl/min. To measure the kinetic parameters, various concentrations of OsGEN-L were loaded for 120 s, and the apparent equilibrium constants (K_D) for OsGEN-L-OsPCNA were calculated from the association and dissociation curves of the sensorgrams, using the BIAevaluation programme (GE Healthcare).

Endonuclease assays

The reaction mixture (40 µl) contained 25 mM Tris–HCl, pH 7.5, 5 mM $MnCl_2$, 25 mM KCl, 1 mM DTT, 10 µg/ml BSA, 0.1% Nonidet P-40, 10% glycerol, 5 nM ^{32}P-labelled 5′-flap DNA substrate and His-tagged OsGEN-L protein (as indicated). In the case of HJ resolvase assay, KCl was added to 150 mM. The reactions were performed at 30°C for 2 h, and were stopped by adding 40 µl phenol:chloroform: isoamyl alcohol (PCI, 25:24:1, pH 7.9). The reaction products were fractionated by 12% native polyacrylamide gel electrophoresis (PAGE) in TAE or 12% denaturing (8 M urea) PAGE in TBE (89 mM Tris–borate, 1 mM EDTA), followed by autoradiography. The results were obtained with a Typhoon TRIO$^+$ variable mode imager and were quantified by the ImageQuant TL analysis software (GE Healthcare).

Determination of exact cleavage site in HJ DNA

The synthetic HJ composed of HJ-1, -2, -3 and -4, having uniquely 5′-^{32}P-labelled arm were incubated with OsGEN-L in the reaction solution at 30°C for 2 h. The reaction mixture was subjected to phenol extraction, and the loading buffer (98% deionized formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) was added. These solutions were loaded onto 12% denaturing PAGE (containing 8 M urea). The size marker was prepared by the GA reaction of Maxam-Gilbert method for each labelled oligonucleotide.

Ligation of the HJ cleavage products

The experiment for the cleavage and re-ligation of the HJ DNA by OsGEN-L was performed basically as described in our previous study for Hjc (*31*). An HJ DNA with asymmetric arm lengths was prepared by annealing four oligonucleotides, HJm-1, HJm-2 (59), HJm-3 (59) and HJm-4. The HJ DNA (10 nM) was incubated with or without OsGEN-L (1 µM) in 100 µl at 30°C for 2 h and was stopped by phenol. The DNA was precipitated by ethanol and dissolved in ligation buffer. Taq DNA ligase (New England Biolabs) was added and incubated at 45°C for 30 min. The products were analysed in 12% denaturing PAGE followed by autoradiography.

Gel-retardation assay

The 5′-^{32}P-labelled ssDNA, dsDNA, 5′-flap and HJ-structured DNA (5 nM) were used to test the DNA binding ability of OsGEN-L. The reaction mixtures (20 µl), containing 25 mM Tris–HCl, pH 7.8, 5 mM $MnCl_2$, 25 mM KCl, 1 mM DTT, 10 µg/ml BSA, 0.1%

NP-40, 10% glycerol, 5 mM EDTA, 5 nM 5′-^{32}P-labelled DNA substrate and OsGEN-L were incubated at 30°C for 20 min. Loading buffer (5×), containing 20 mM HEPES–NaOH, pH 7.5, 50% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol, was added to the reaction mixtures and an aliquot (7 µl) was fractionated on a 4% polyacrylamide gel in 1 × TAE (40 mM Tris–acetate, and 1 mM EDTA) by electrophoresis at 15 mA for 40 min, and then visualized by an imager (Typhoon TRIO$^+$, GE Healthcare).

Results

Overproduction and purification of OsGEN-L

We cloned the cDNA of the gene encoding OsGEN-L by a PCR amplification method from the *O. sativa* cDNA library, as described in the 'Materials and Methods' section. The PCR-amplified DNA was inserted into a modified pET21a vector, in which the OsGEN-L gene is expressed as the His-tagged protein. The expression plasmid was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL, and the expression of the target gene was induced by IPTG at 16°C. About 1.6 mg of the His-tagged OsGEN-L protein was purified to near homogeneity from a 4 litre culture of *E. coli* cells (Fig. 1A). A gel filtration analysis revealed that the purified OsGEN-L protein eluted at the volume corresponding to the molecular weight of 86,600, calculated from the standard curve based on the size marker proteins (Fig. 1B and C). This result suggests that OsGEN-L exists as a monomer in solution. The theoretically calculated molecular weight of OsGEN-L from the deduced amino acid sequence is 73,100. The reason why the molecular weight from the gel filtration analysis is higher than the theoretical value may be that the OsGEN-L protein contains an intrinsically disordered region, which was predicted from the amino acid sequence by the IUPred software, as shown in Fig. 1D.

Substrate specificity of OsGEN-L

OsGEN-L has a FEN activity, as reported previously (*18*). However, in that report, the FEN activity was quite weak, as compared with that of OsFEN-1 in the parallel experiment. Therefore, to determine the substrate specificity of OsGEN-L in more detail, we tested various substrates, including a 5′-flap DNA. The purified OsGEN-L protein was incubated with synthetic DNA substrates with various structures, after labelling each 5′-terminus with ^{32}P, and the reaction mixtures were analysed by polyacrylamide gel electrophoresis. As shown in Fig. 2, OsGEN-L showed high specificity for the 5′-flap structure, while a fork-like structure, which is most similar to the 5′-flap structure, was also cleaved, but much less efficiently.

Biochemical properties of the FEN activity of OsGEN-L

To optimize the reaction conditions for the FEN activity of OsGEN-L, reactions were performed under different pH and temperature conditions. OsGEN-L showed the highest activity as a FEN at 30°C and pH 7.8 (Fig. 3A and B). The metal ion dependency was also investigated. Various metals, including Mn^{2+}, Mg^{2+}, Zn^{2+} or Ca^{2+}, were added to

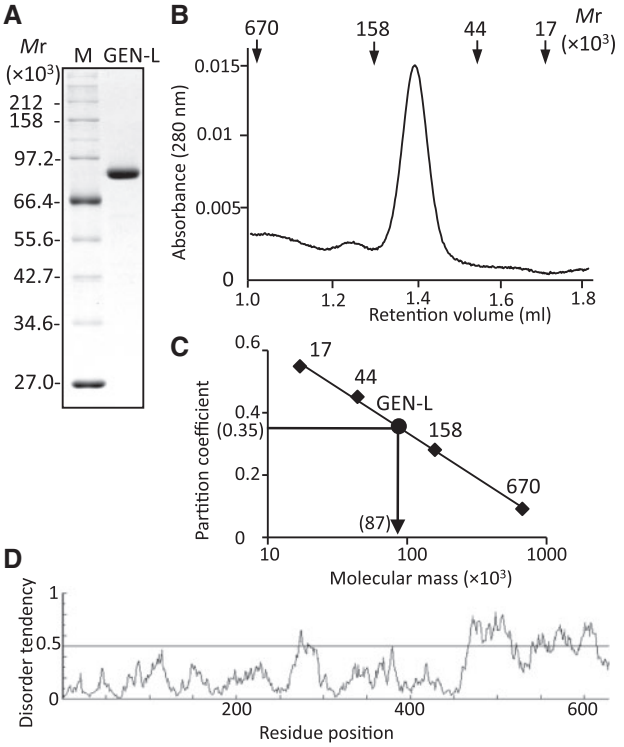

Fig. 1 Preparation of purified His-tagged OsGEN-L. (A) The purified OsGEN-L protein (2.0 µg) was subjected to 10% SDS–PAGE, and the gel was stained by Coomassie Brilliant Blue (CBB). M, molecular mass standards (New England Biolabs Inc.). (B) The oligomeric state of OsGEN-L in solution was analysed by gel filtration, using a Superdex 200 3.2/30 column. The elution profiles, monitored by the absorbance at 280 nm, are shown. The standard curve was obtained with marker proteins on a parallel chromatography. The peak positions of the marker proteins are indicated on the top. (C) The apparent molecular weight of OsGEN-L was calculated from the standard curve of the chromatography. (D) Disorder profile plots for OsGEN-L. The amino acid sequence of the full-length OsGEN-L (629 amino acids) is plotted against the probability of intrinsic disorder. The horizontal line indicates the order/disorder threshold of 0.5.

the reaction buffer, and the cleavage efficiencies were compared. The cleavage reaction progressed well in the presence of Mn^{2+} or Mg^{2+}, but Ca^{2+} and Zn^{2+} could not substitute for Mn^{2+} or Mg^{2+}. Therefore, the concentration dependence was examined for the Mn^{2+} and Mg^{2+} ions. As shown in Fig. 3C, Mn^{2+} processed the reaction much more efficiently than Mg^{2+}. The Mn^{2+}-stimulated property was also reported previously for the mouse FEN-1 endonuclease (*35*); however, DmGEN from *Drosophila melanogaster* prefers Mg^{2+}, rather than Mn^{2+}, for its FEN activity (*17*). The salt test indicated that OsGEN-L is sensitive to the salt concentration in the reaction mixture, and the reaction progresses more efficiently at concentrations <50 mM. There were minimal differences between K^+ and Na^+ (Fig. 3D). As commonly used additives, BSA and detergent were also tested (data not shown). The final optimum reaction mixture for the OsGEN-L FEN activity comprised 25 mM Tris–HCl, pH 7.8, 5 mM $MnCl_2$, 25 mM KCl, 1 mM DTT, 10 µg/ml BSA, 0.1% Nonidet P-40 and 10% glycerol.

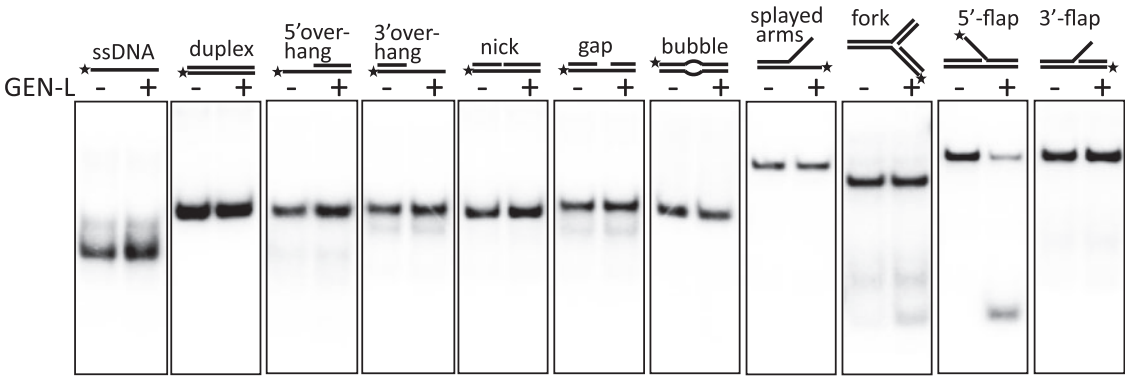

Fig. 2 Substrate specificity of OsGEN-L nuclease activity. DNA substrates with 11 different structures (schematically shown on the top) were prepared with 5′-^{32}P end-labelling. These DNAs (5 nM) were incubated in the presence or absence of OsGEN-L (150 nM) at 30°C for 120 min. The reactions were stopped by PCI [Phenol: Chloroform: Isoamyl alcohol = 25:24:1 (pH 7.9)] extraction. The reaction products were resolved by 12% native PAGE, and the gel electrophoresis images were obtained by autoradiography.

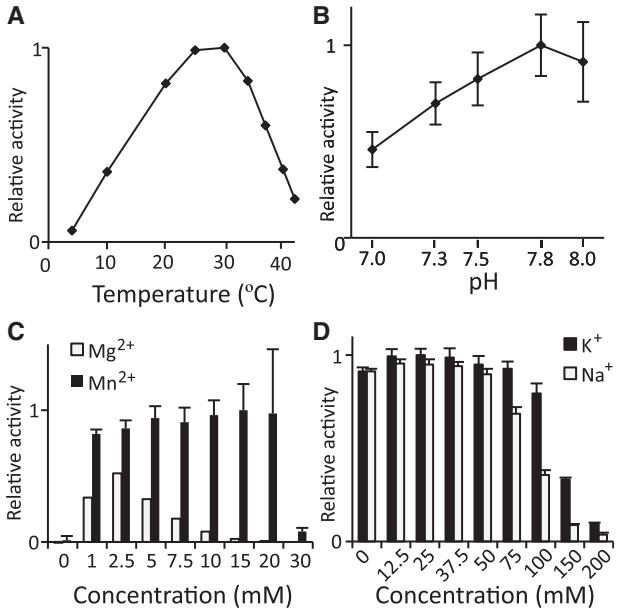

Fig. 3 Biochemical properties of the FEN activity of OsGEN-L. (A) Effect of temperature. OsGEN-L (180 nM) and a 5′-^{32}P-labelled 5′-flap DNA (5 nM) were incubated in the reaction solution (25 mM Tris–HCl, pH 7.8, 5 mM $MnCl_2$, 25 mM KCl, 1 mM DTT, 10 μg/ml BSA, 0.1% Nonidet P-40, 10% glycerol) for 2 h at the indicated temperatures, and the relative cleavage efficiencies were plotted. (B) pH dependence. OsGEN-L (180 nM) and 5′-^{32}P-labelled 5′-flap DNA (5 nM) were incubated in reaction buffers at different pH values, containing 5 mM $MnCl_2$ and 25 mM KCl. (C) Divalent metal dependence of OsGEN-L. The 5′-flap cleavage reaction was performed in solutions with different concentrations of $MnCl_2$ or $MgCl_2$ (pH 7.8, 25 mM KCl). (D) Salt dependence. The 5′-flap cleavage reaction was performed in solutions with different concentrations of KCl or NaCl (pH 7.8, 5 mM $MnCl_2$). For all of the analyses, the reaction products were subjected to 12% native PAGE, and the gel images were obtained by autoradiography.

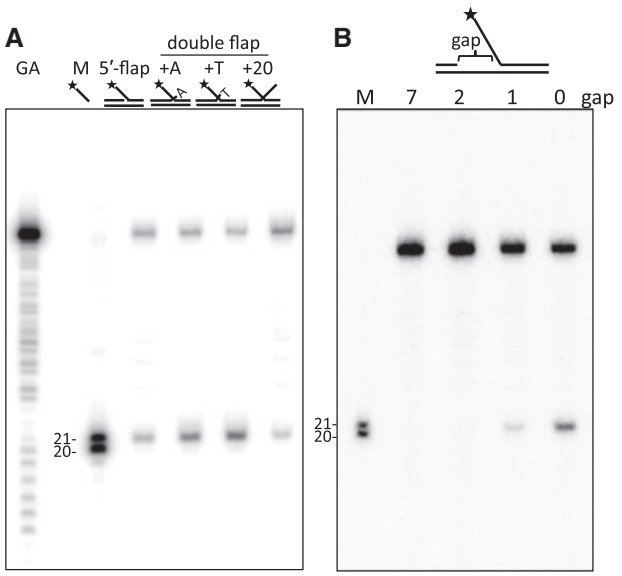

Fig. 4 Endonuclease activity on 5′-flap structured DNA. (A) OsGEN-L cleaved the 5′-flapped DNA strand at 1 nt next to the 3′-end from the junction. His-tagged OsGEN-L (150 nM) and 5′-^{32}P-labelled 5′-flap or double-flap DNA (5 nM) were incubated in a reaction solution at 30°C for 2 h. The reaction products were analysed by denaturing PAGE alongside a GA sequence ladder produced by a Maxam-Gilbert reaction of the oligonucleotide used for the 5′-labelling. M is the size markers containing 5′-^{32}P-labelled oligonucleotides with 20 and 21 nt lengths, with the same sequence as the flapped DNA. (B) Effect of a gap at the branch point of the flapped DNA on the FEN activity. Lanes 0, 1, 2 and 7 represent gap lengths of 0, 1, 2 and 7 nt. M is the size markers of 20- and 21-nt long DNA fragments.

Structural preference of the FEN activity of OsGEN-L

To characterize the endonuclease activity of OsGEN-L on 5′-flap DNA, 5′-^{32}P-labelled 5′-flap, gapped flap and double-flap substrates were used to compare the cleavage efficiency. OsGEN-L cleaved the 5′-flap at 1 nucleotide next to the 3′-end from the junction (Fig. 4A), which is a property shared with the other RAD2/XPG family members, such as DmGEN (*17*) and HsGEN1 (*26*). The cleavage efficiency of the 5′-flapped DNA was enhanced with double-flapped DNAs with one nucleotide as the 3′-flap; however, a longer 3′-flap was not suitable in the double-flapped DNA (Fig. 4A). OsGEN-L showed weak activity for the flap with a 1-nt gap at the junction, and the activity decreased further as the gap became wider (Fig. 4B), in a similar manner to DmGEN (*17*). OsGEN-L also showed cleavage activity on the template strand for lagging synthesis of the replication fork-like structure, containing a gap on the lagging strand synthesis

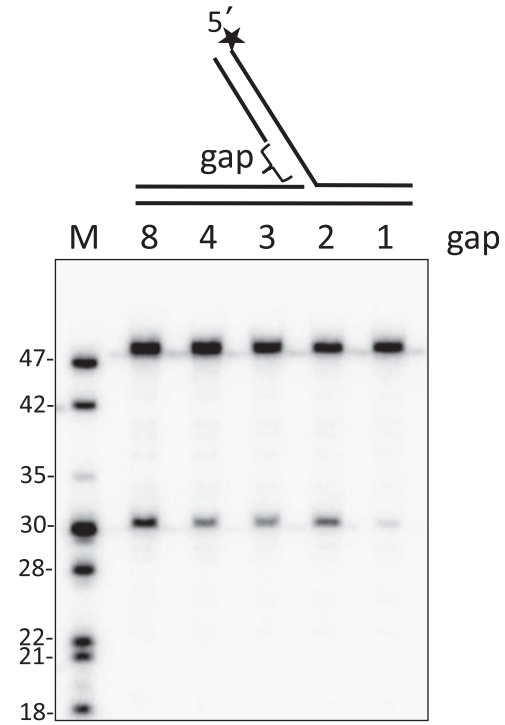

Fig. 5 Effect of a gap in the replication fork-like structure on the nuclease activity of OsGEN-L. The 5′-^{32}P-labelled fork-like DNA, with various gap lengths at the junction (each 5 nM), was incubated with OsGEN-L (100 nM) at 30°C for 120 min. The reaction products were analysed by native PAGE, followed by autoradiography.

(blocked 5′-flap) (Fig. 5). The cleavage site was the same as that on the 5′-flap DNA. These properties are conserved between OsGEN-L and DmGEN (*17*).

Interaction between OsGEN-L and OsPCNA

OsGEN-L showed high specificity for 5′-flap structured DNA, and therefore, it may function with PCNA on the DNA strands, as in the cases of the FEN-1 proteins from other organisms reported previously (*9*). To investigate whether OsGEN-L binds PCNA, SPR analysis was performed, using purified OsGEN-L and OsPCNA. As shown in Fig. 6A, the resonance units (RUs) increased when OsPCNA was immobilized on the Biacore CM5 sensor chip and increasing concentrations of OsGEN-L were passed over it. The calculated K_D from this experiment was 6.41×10^{-7} M, indicating that OsGEN-L directly binds OsPCNA with high affinity and they work together on the DNA. We then investigated the effect of PCNA on the flap endonuclease activity of OsGEN-L *in vitro*. As shown in Fig. 6B, the cleavage activity was clearly stimulated in the presence of PCNA, in a concentration-dependent manner. PCNA interacting proteins (PIP) possess a consensus sequence motif, called the PIP box, which is generally responsible for binding to PCNA. We searched for the PIP box-like sequence in the amino acid sequence of OsGEN-L, and found a candidate in the C-terminal region of the OsGEN-L protein (Fig. 6C). OsGEN-L probably binds OsPCNA at this region. Further mutational analyses will be required to confirm this binding site.

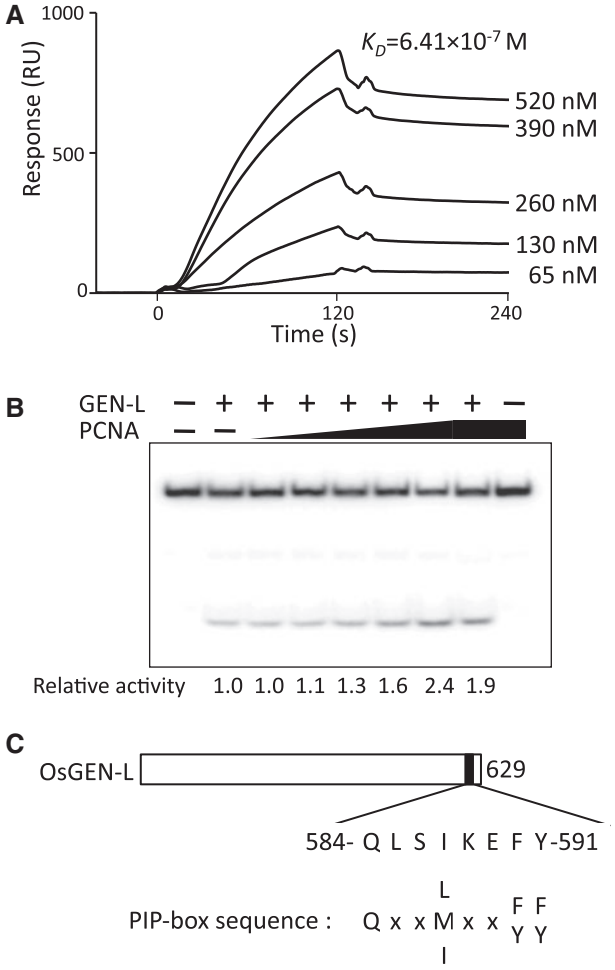

Fig. 6 Interaction between OsGEN-L and OsPCNA. (A) SPR analyses were performed using a BIACORE system, to analyse the physical interaction between the OsGEN-L and OsPCNA proteins. Purified OsPCNA was immobilized on CM5 sensor chips, and various concentrations of OsGEN-L were injected for 120 s. (B) OsPCNA stimulated the FEN activity of OsGEN-L *in vitro*. OsGEN-L (165 nM) and serial concentrations of OsPCNA (0.25, 0.5, 0.75, 1, 1.5 and 2 μM) were incubated in the endonuclease reaction at 30°C for 120 min. The band intensities of the cleaved product were quantified with the image analyser, and the cleavage efficiencies in each reaction relative to that without PCNA are shown at the bottom. (C) Location of the PIP box-like sequence in OsGEN-L. The black box in the OsGEN-L protein indicates a PIP box-like sequence. The consensus sequence of the PIP box is indicated at the bottom.

HJ cleavage activity of OsGEN-L

In addition to the characterization of the FEN activity, a major interest is whether OsGEN-L, like the human GEN1 and yeast Yen1 proteins, possesses HJ resolvase activity. To investigate the HJ cleavage activity of OsGEN-L, 5 nM 5′-^{32}P-labelled four-way junction structured DNA was incubated with OsGEN-L under various reaction conditions, and we detected the activity under one set of conditions (Fig. 7). The HJ cleavage was not observed under the same conditions as those used for the FEN. For the HJ cleavage reaction, high salt (150 mM KCl) was critical. The reason why the optimal salt concentration is different between the FEN and HJ resolvase activities of the same protein is

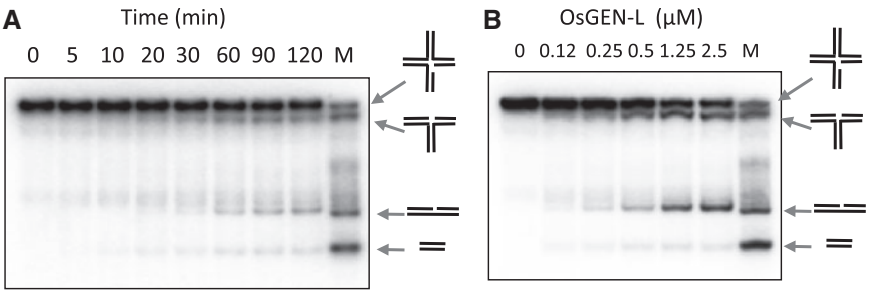

Fig. 7 HJ cleavage activity of OsGEN-L. The four-way junction DNA was prepared by annealing of HJ-1, -2, -3 and 5′-^{32}P-labelled HJ-4, and this DNA substrate (5 nM) was incubated with OsGEN-L (500 nM) at 30°C in a reaction buffer and the aliquots were picked up after 5, 10, 20, 30, 60, 90 and 120 mins (A). The same reaction was done with various amounts of OsGEN-L from 0.125 to 2.5 μM at 30°C for 120 min (B). The reaction products were separated by 12% native PAGE, and were visualized by autoradiography. The marker DNAs loaded on the right side were prepared by annealing of the appropriate oligonucleotides after ^{32}P-labelling of 1 nt.

not clear. The four-way junction structure can be stabilized in the solution with a high salt concentration, and the structure may be critical for the enzyme recognition. The gel electrophoresis revealed three bands as reaction products, corresponding to the fork-structure, nicked duplex and duplex DNA (one arm of the HJ DNA), respectively, as confirmed by each structured DNA labelled by ^{32}P after construction by annealing of the oligonucleotides with appropriate combinations (loaded on the right side of the gel as the marker DNAs). This result indicates that OsGEN-L can cleave one branched duplex arm from the HJ (asymmetrical cleavages of the two of the four strands), in addition to the common property of the HJ resolvase to cleave the junction point symmetrically, to produce the nicked duplex. These two types of cleavages seem to happen simultaneously, but not sequentially, from the observation that the nicked three-way junction and nicked duplex appeared at almost the same time in the time course and dose dependent experiments (Fig. 7). However, four sites at the junction were not equally accessed by OsGEN-L, because the ^{32}P-labelled arm duplex was not detected in the reactions. The exact cleavage sites for each strand were determined by denaturing gel electrophoresis. As shown in Fig. 8, a major band was detected from the strand HJ-1, -2 and -4. The cleavage efficiency was less in the strand HJ-3, as compared with other three strands. This result shows that the main cleavage sites of OsGEN-L are located between the first and second nucleotides from the junction centre for each strand. One more important property of the HJ resolvase is that it provides the products that can be rejoined by ligation. To respond this question, we performed a cleavage–religation experiment using a movable HJ with the arms of asymmetrical lengths as a substrate for OsGEN-L, as previously used for archaeal Hjc (*31*). Since the junction point is movable in the substrate HJ, the visible (^{32}P-labelled) products were expected to be from 30- to 43-mer. As shown in Fig. 9, several distinct bands were produced by the reaction with OsGEN-L, and some of the major products (31-mer) were converted to a longer strand (70-mer) by treatment with Taq DNA ligase, which has a nick ligation, but not end joining, activity. This result indicates that the nicked duplex produced by OsGEN-L cleavage at symmetrically related sites of the two strands leave 5′-phosphate and 3′-hydroxyl termini, respectively, as conserved in the HJ resolvases. However, OsGEN-L also cleaves the HJ DNA in an asymmetrical mode as described above. The arm duplex and the nicked three-way junction, produced by asymmetric nicking in the two cleavage sites, are not ligatable, even though the cleavage leaves 5′-phosphate and 3′-hydroxyl termini (some of the produced nicked three-way junctions are ligatable, although they are not visible because of non-labelled). The observed bands by OsGEN-L cleavage probably include these non-ligatable products. Further analyses are required to assign all the observed bands precisely. However, the presented data support that OsGEN-L resolve the synthetic HJ into a pair of duplex DNA strands *in vitro*, like other HJ resolvases.

Binding of OsGEN-L to DNAs with various structures

To investigate the structural preference as the substrate for OsGEN-L, we performed an *in vitro* gel-retardation assay using ^{32}P-labelled DNA as probes in the presence of 5 mM EDTA to prevent cleavage reactions. As shown in Fig. 10, OsGEN-L obviously bound to a 5′-flap and a HJ DNAs with higher affinity as compared with normal single-stranded and double-stranded DNAs. This result supports the substrate specificity of OsGEN-L presented in this study. Furthermore, the HJ DNA was slightly more preferable than the 5′-flap DNA for OsGEN-L even in the low salt condition. It is still not known whether this difference of affinity *in vitro* causes any effect on the physiological functions of OsGEN-L, and further analyses are required to understand the roles of this multifunctional enzyme.

Discussion

We characterized the enzymatic properties of OsGEN-L, using the highly purified protein, and detected both the FEN and HJ resolvase activities. A previous report demonstrating the *in vitro* FEN activity used an OsGEN-L protein with an S-tag at the N-terminus and His-tags at the N-terminus and C-terminus, respectively (*18*). Our protein has only

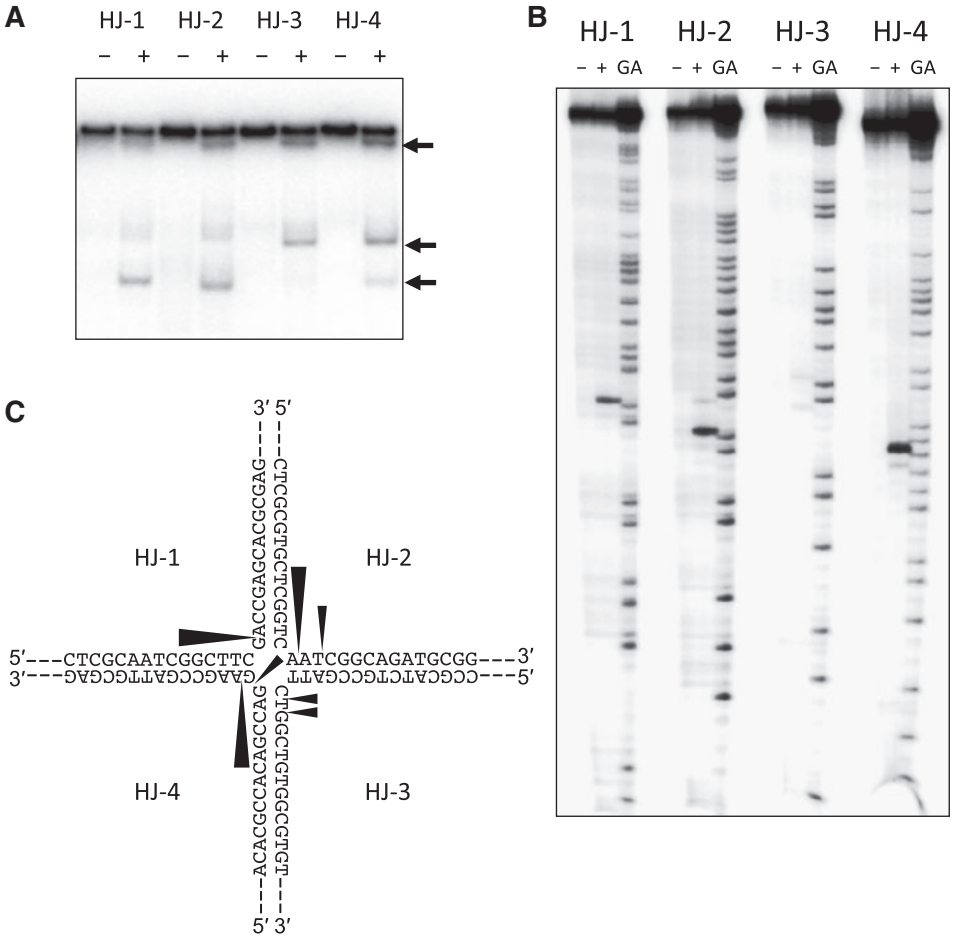

Fig. 8 Determination of exact cleavage site in HJ DNA. The synthetic HJ DNAs including one 5′-^{32}P-labelled strand of the four oligonucleotides were used as substrates and the cleavage reactions were done at 30°C for 120 min. The reaction products were analysed in 12% native PAGE, followed by autoradiography (A). The cleavage products are indicated by arrows. The same reactions were subjected to 12% denaturing PAGE (B). Both in (A) and (B), 5′-^{32}P-labelled strands were shown on the top, and − and + indicate the reactions without and with OsGEN-L, respectively. GA in (B) indicates the GA ladders produced by Maxam-Gilbert methods for each 5′-^{32}P-labelled strand. The cleavage sites obtained from (B) are represented in the HJ sequence by arrowheads with cutting efficiencies by their sizes (C). OsGEN-L cleaved HJ at the junction sites located 1 nt to the 3′ side of the branch point.

a His-tag at the N-terminus, and this structural difference may affect some of the biochemical properties. The FEN activity was clearly shown in our protein, as compared with the previous report, in which only very weak activity was detected (*18*). The properties of OsGEN-L shown in this study suggest that this enzyme may function to remove primer regions for Okazaki fragment maturation during lagging strand synthesis. In eukaryotic DNA replication, FEN-1 primarily functions in primer removal. *O. sativa* has two other FEN-1 nucleases, designated as OsFEN-1a and OsFEN-1b (*36*). Their deduced amino acid sequences revealed a high degree of similarity. The FEN activity of OsFEN-1a, as well as its physical and functional interactions with PCNA, was characterized in detail (*37*), and furthermore, OsFEN-1a can partially complement the deficiency of the *rad27* gene, encoding FEN-1 in *S. cerevisiae* cells. On the other hand, the enzymatic activity of OsFEN-1b has not been characterized, and it does not complement the function of FEN-1 in yeast cells (*36*). Therefore, the function of OsFEN-1b has not been definitively elucidated. However, it should be involved in some aspect of DNA metabolism, as the authors discussed, because its gene is preferentially expressed in proliferating tissues, such as the shoot apical meristem and young leaves (*36*). One of the interesting differences between OsFEN-1a and OsFEN-1b is that OsFEN-1b lacks a PIP box-like sequence at the C-terminal region. OsFEN-1b may function independently of PCNA. In this report, we showed that OsGEN-L interacts with OsPCNA. Another interesting issue is that two PCNAs exist in *O. sativa* (*37*), but only one has been studied so far (*38*). The relationships between these structure-specific endonucleases and PCNAs are also interesting research subjects in rice DNA replication and repair. A similar nuclease, OsEXO1, which is a homolog of the class III exonuclease 1, has been reported in *O. sativa* (*39*). The gene encoding OsEXO1 is preferentially expressed in meristematic tissues and panicles, and OsEXO1 can partially complement the deficiency of the *rad27* gene in *S. cerevisiae* cells, although the enzyme has not been biochemically characterized (*39*). These results are the same as those for OsFEN-1a, as described above. It would be interesting to elucidate how these multiple enzymes possessing the

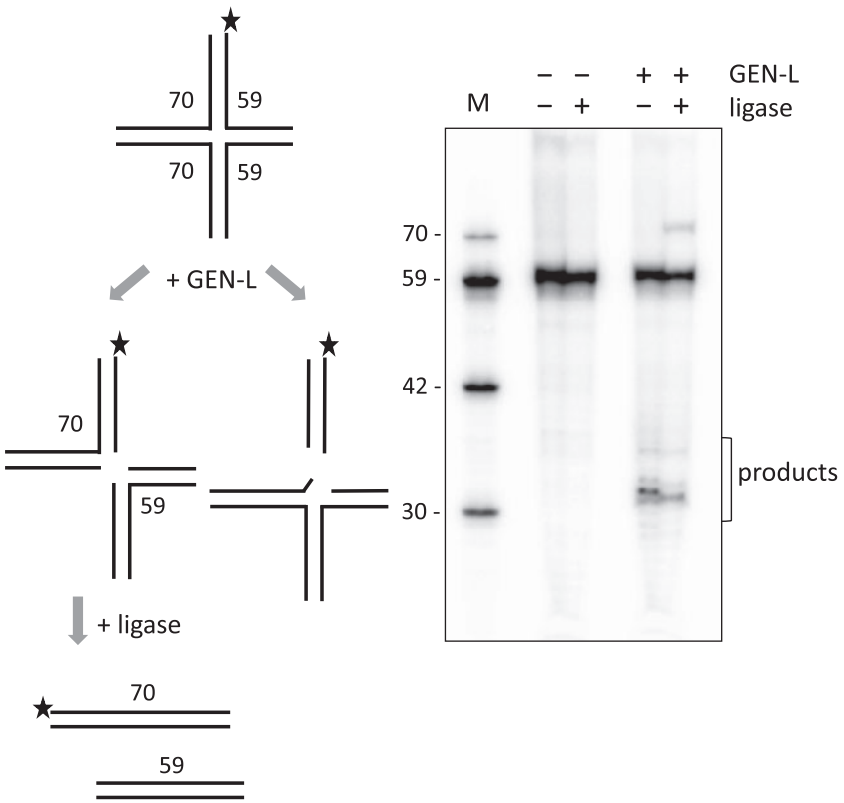

Fig. 9 Re-ligation of the cleavage products from HJ DNA. Schematic representation of the reaction is shown on the left. The ^{32}P-labelled (marked by asterisk) short strand (59 nt in length) was cleaved by OsGEN-L to produce the nicked duplex DNA (left) and the arm duplex (right) by symmetrical and asymmetrical cleavages, respectively. Ligation of the nicks results in elongation of the labelled strand to 70 nt. The arm duplex is not ligatable. These reactions were performed as described in 'Materials and Methods' section. The products were visualized by autoradiography following denaturing (8 M urea) PAGE. Incubation with DNA ligase led to the formation of a band corresponding to 70 nt in length.

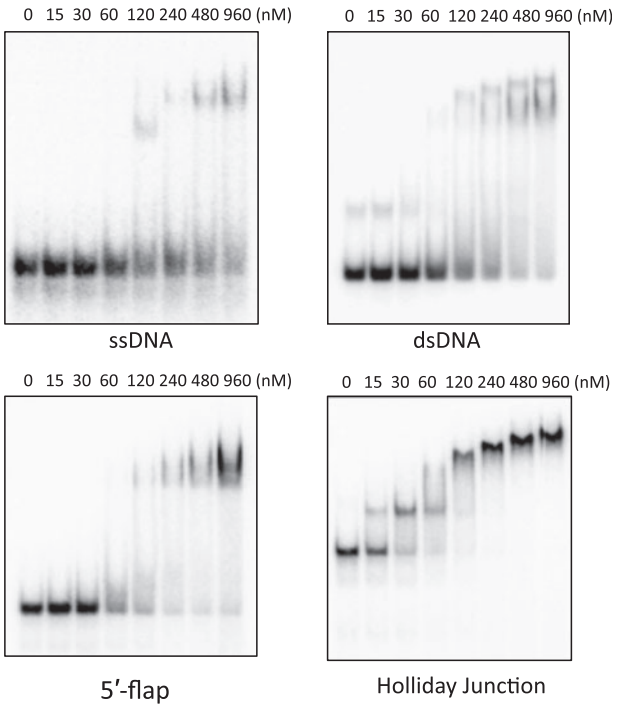

Fig. 10 DNA binding preference of OsGEN-L. The OsGEN-L proteins was incubated with a 49 nt ^{32}P-labelled single-stranded DNA, a double-stranded DNA, a 5′-flap DNA, and a HJ DNA, respectively, at 30°C for 20 min, and the reaction products were analysed by 4% PAGE. Increasing amounts of OsGEN-L (shown on the top of each panel) led to the band shift of DNA indication the complex formations of OsGEN-L-DNA.

FEN activity share the functions of DNA metabolism in rice cells.

In addition to the FEN activity, OsGEN-L has HJ resolution activity, as shown by the homologs from human (GEN1) and yeast (Yen1). It was interesting to discover that human GEN1 exists as a monomer in solution (*26*), because all of the known HJ resolvases form stable homodimers, and each subunit cleaves one of the two junction sites to produce a pair of nicked duplexes, which can be sealed by DNA ligase (*29*, *31*). Our results showed that OsGEN-L also exists as a monomer in solution, and does not form a stable dimer. The cleavage reaction of the HJ DNA by OsGEN-L generated three different product bands, which were determined to be a nicked three-way junction, a nicked duplex, and a duplex with the length of one of the four arms. Therefore, we predicted that the OsGEN-L monomer accesses the junction sites and cleaves one strand individually. When the OsGEN-L symmetrically accesses two of the four-junction sites, the reaction product should be a pair of nicked duplexes, as with other HJ resolvases. We confirmed that a nicked duplex was rejoined *in vitro* by the DNA ligase (Fig. 9), as previously shown for human GEN1 (*26*). Two OsGEN-L molecules can also asymmetrically access the two junctions, which can divide the HJ into a nicked three-way junction and an arm duplex DNA. It is possible that an HJ containing a nick in one

of the four strands, which cannot be detected by our assay system, was produced by OsGEN-L, although human GEN1 does not generate such a reaction product (*26*). We used a synthetic HJ DNA with a 35 nt length for each branched strand, and therefore, the observed asymmetrical cleavage may be artificial, and these two kinds of products may not actually be generated in rice cells. The angles between the branched arm strands are usually not even, but the HJ has a preferred structure. The symmetrical cleavage of the HJ by OsGEN-L may be controlled *in vivo* by the environmental conditions to adjust only the symmetrical access to the junction for OsGEN-L, if it actually works as an HJ resolvase in the cells. The cleavage preferences of OsGEN-L for the HJ should be further analysed. In addition, we have been analysing whether OsPCNA stimulates the HJ cleavage activity of OsGEN-L; however, we cannot find the condition to show the PCNA-dependent stimulation of HJ resolvase activity *in vitro* so far. The answer of this issue should be obtained from further experiments.

In conclusion, we confirmed that OsGEN-L has a FEN activity *in vitro* and characterized its activity in detail, including its stimulation in a PCNA-dependent manner. In addition, like other GEN homologs, OsGEN-L has HJ resolvase activity (*28*). These results support the proposal that OsGEN-L is an important enzyme involved in multiple DNA repair processes in rice cells.

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Conflict of interest

None declared.

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