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Arabidopsis ENDO2: Its Catalytic Role and Requirement of N-Glycosylation for Function

Chia-Yun Ko,[†,‡] Yi-Ling Lai,[§] Wen-Yu Liu,[†,#] Chia-Hui Lin,[†] Yu-Ting Chen,[⊥,Δ] Long-Fang O. Chen,[§] Tsai-Yun Lin,*[,†] and Jei-Fu Shaw*[,‡,∥,Δ]

[†]Institute of Bioinformatics and Structural Biology, National Tsing Hua University, Hsinchu, 30013, Taiwan

[‡]Agricultural Biotechnology Research Center and [§]Institute of Plant and Microbial Biology, Academia Sinica, Taipei, 11529, Taiwan

[⊥]Insititute of Genomics and Bioinformatics and [Δ]Agricultural Biotechnology Center, National Chung Hsing University, Taichung, 40227, Taiwan

[∥]Department of Biological Science and Technology, I-Shou University, Kaohsiung City 84001, Taiwan

Supporting Information

ABSTRACT: The *Arabidopsis thaliana* At1g68290 gene encoding an endonuclease was isolated and designated ENDO2, which was cloned into a binary vector to overexpress ENDO2 with a C-terminal 6 × His-tag in *A. thaliana*. Our *Arabidopsis* transgenic lines harboring *35SP::ENDO2* produced stable active enzyme with high yield. The protein was affinity purified from transgenic plants, and its identity was confirmed by liquid chromatography−mass spectrometry and automatic Edman degradation. ENDO2 enzyme digests RNA, ssDNA, and dsDNA, with a substrate preference for ssDNA and RNA. The activity toward ssDNA (361.7 U/mg) is greater than its dsDNase activity (14.1 U/mg) at neutral pH. ENDO2 effectively cleaves mismatch regions in heteroduplex DNA containing single base pair mismatches or insertion/deletion bases and can be applied to high-throughput detection of single base mutation. Our data also validated that the removal of sugar groups from ENDO2 strongly affects its enzymatic stability and activity.

KEYWORDS: *N-glycosylation, ENDO2, single-strand-preferring nuclease, mismatch specific endonuclease, programmed cell death*

INTRODUCTION

Individual nucleases show preferences for various structures of DNA and RNA. Some nucleases prefer double-stranded polymers, while others prefer single-stranded polymers. Nuclease can be divided to DNase, RNase, and S1/P1-type endonuclase.[1] S1 nuclease from *Aspergillus oryzae*[2,3] and P1 nuclease (EC 3.1.30.1) from *Penicillium citrinum*,[4−6] which possess high specificity for single-stranded DNA (ssDNA) and RNA and low activity for double-stranded DNA (dsDNA), were also classified as single-strand-specific nucleases or single-strand-preferring nucleases.[1,7,8] Plant S1/P1 type endonucleases can be categorized, based on their cofactor preference, as either zinc- or calcium-dependent.[1] Zinc-dependent endonucleases show a pH optimum in the acidic range and substrate preference of RNA > ssDNA > dsDNA. Most calcium-dependent endonucleases exhibited a pH optimum in the neutral range and substrate preference of ssDNA > RNA > dsDNA.

The structure of P1 nuclease reveals an active site that coordinates three zinc ions as a cofactor for catalysis in a pocket. Near the active site, there are two mononucleotide binding sites for substrate binding.[9,10] The nine zinc ion-interacting amino acids are highly conserved in all zinc-dependent endonucleases.[11] Complete removal of zinc ions with EDTA causes the loss of P1 nuclease activity.[5] Zinc ions are important not only in catalysis but also in stabilizing protein structure. *N*-Glycan attaches at Asn92, one of the four N-glycosylation sites, forming a hydrophilic pocket to avoid the exposure of Trp55 to solvent.[9,10]

Plant single-strand-specific endonucleaseses exhibit high selectivity for the single-stranded region in dsDNA.[8] For instance, endonuclease 1 (ENDO1) and CEL1 enzymes have been demonstrated to cleave bulge loops in heteroduplex DNA with no nucleotide preference, and they cleaves all types of mismatches with a high efficiency.[12−14] ENDO1 and CEL1 have been applied to high-throughput detection of single base mutations by targeting induced local lesions in genomes (TILLING) for mutants screening, which has been used to screen random mutants induced by ethyl methane sulfonate (EMS) in *Arabidopsis*,[15] barley,[16] and wheat[17] and *N*-methyl-*N*-nitrosourea (MNU) in rice[18] to find mutations in a target gene. The identified sequence information of the mutant can be linked to the biological function of the gene.

The *Arabidopsis* ENDO1 (also named bifunctional nuclease 1, BFN1) is induced during leaf and stem senescence.[11] In addition to leaf senescence, plant ENDOs are involved in different forms of programmed cell death (PCD),[19] including tracheary element differentiation,[20,21] aleurone cell death,[22] endosperm development,[23] and hypersensitive response.[24] The protein sequence of ENDO1 is highly similar to the CEL1 from *Apium graveolens* and the PCD-associated ZEN1 nuclease from *Zinnia elegans*. ZEN1 plays a requisite role in nuclear DNA degradation during PCD associated with xylem development.[21]

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Arabidopsis thaliana genome contains five genes encoding zinc-dependent ENDOs, *ENDO1* (At1g11190), *ENDO2* (At1g68290), *ENDO3* (At4g21590), *ENDO4* (At4g21585), and *ENDO5* (At4g21600). Up to the present, only the properties of ENDO1 enzyme have been characterized. The functions of other ENDO members remain unclear. This study reports the cloning and expression of *ENDO2* gene via a homologous transgenic system and the characterization of ENDO2 protein purified from *Arabidopsis* transgenic plants. We found that N-glycosylation is crucial for ENDO2 enzyme activity. ENDO2 has the potential to detect single base pair mismatches and can be used to screen random mutants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. *A. thaliana* (Columbia ecotype) plants were grown under long-day conditions (16 h light/8 h dark) at 22 °C with light irradiance of 150 μmol m^{-2} s^{-1} and relative humidity of 65%. Seeds were surface-sterilized in 33% commercial bleach and 0.1% Triton X-100 solution for 15 min. After stratification at 4 °C for 2−3 days, seeds were plated in Petri dishes with 1/2 × Murashige−Skoog medium containing 0.11 g L^{-1} B5 vitamins, 1% sucrose, and 0.8% Phyto agar (pH 5.7). Ten day old kanamycin-resistant (50 mg L^{-1}) transgenic seedlings were planted in soil. After the siliques wilted, seeds were collected and stored at 4 °C.

Isolation of RNA and Cloning of ENDO cDNAs. Total cellular RNA was extracted[25] from a 1 g sample and finally dissolved in 100 μL of diethylpyrocarbonate-treated H_2O (DEPC-H_2O). Poly(A) mRNA was isolated using the PolyATract mRNA isolation system III (Promega, WI), and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, CA). The *ENDO* genes were obtained by polymerase chain reaction (PCR) using primer pairs corresponding to and complementary with the *ENDO1* (At1g11190), *ENDO2* (At1g68290), and *ENDO3* (At4g21590) cDNA sequences. Each of the PCR-amplified DNA fragments was cloned into pGEM-T easy vectors and confirmed by sequencing.

Overexpression of ENDO1/2 in *Arabidopsis* Plants. The *ENDO1/2* cDNAs were PCR-amplified with primers to include a C-terminal 6 × His tag (Table S1 in the Supporting Information), cloned into pBI121 plasmid to form 35SP::ENDO1/2-HisOE, and introduced into *Agrobacterium tumefaciens* strain GV3101::pMP90 by electroporation.[26] Transformation into *Arabidopsis* was performed with the vacuum infiltration method (http://transplant.sinica.edu.tw/english/protocol/trans/1.htm). The pBI121 plasmid transformed plants were used as the control. Seeds were placed on selecting medium and the 10 day old kanamycin-resistant plants were transferred to soil. Two week old seedlings (T3 homozygous transgenic plants) were harvested to extract the recombinant ENDO1 and ENDO2 protein for assays of DNase and RNase activity.

Purification of Recombinant ENDO Proteins from Transgenic Plants. Transgenic *Arabidopsis* seedlings overexpressing the ENDO2/ENDO1 protein were weighed and ground into a fine powder in liquid nitrogen with a prechilled mortar and pestle. The powder was homogenized (1 g: 1 mL) with 20 mM Tris buffer (pH 7.4) containing EDTA-free protease inhibitor cocktail (1 tablet for 50 mL) (11 873 580 001, Roche, Germany). The homogenates were spun at 4000*g* for 30 min, and the supernatant was recentrifuged at 22000*g* for 90 min. The supernatant was filtered with 0.45 μM Millipore membrane and loaded into a DEAE column (DEAE Sepharose CL-6B, GE Healthcare, United States), equilibrated, and washed with 3 bed volumes of DEAE wash buffer (20 mM Tris buffer, 25 mM NaCl, pH 7.4). The recombinant ENDO protein was recovered by elution with 3 bed volumes of DEAE-elution buffer (20 mM Tris buffer, pH 7.4, and 50/100/250/500 mM NaCl).

Fractions containing recombinant ENDO protein were analyzed by protein blots hybridized with anti-His tag antibody. The collected fractions were applied to Ni Sepharose 6 Fast Flow (GE Healthcare) columns equilibrated with wash buffer (20 mM Tris buffer, 250 mM NaCl, pH 7.4). After it was washed with 3 bed volumes of His-wash buffer containing 10 mM imidazole, the recombinant protein was eluted with 3 bed volumes of elution buffer (20 mM Tris buffer, 250 mM NaCl, and imidazole from 20 to 250 mM, pH 7.4). The recombinant protein was detected with 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and was excised and eluted.

In-Gel RNase and DNase Activities Assay. The total protein was extracted from shoots of transgenic plants following the method of Yen and Green[27] with some modifications. Approximately 200 mg of tissue was homogenized at room temperature with 200 μL of extraction buffer (20 mM Tris buffer, pH 7.4, 5 mM PMSF). After centrifugation at 13000*g* at 4 °C for 30 min, the soluble protein was quantified by the Bradford assay.[28] The separating gel contained 12% (w/v) PAGE with 2.4 mg mL^{-1} Torula yeast RNA (R-6625, Sigma, MO) or 0.8 mg mL^{-1} ssDNA heat denatured DNA from salmon testes (D-1626, Sigma). After electrophoresis, SDS was washed from the gel for 10 min twice with 25% (v/v) isopropanol and 10 mM Tris buffer (pH 7.0). Isopropanol was removed by soaking in 10 mM Tris buffer twice. After preincubation in 100 mM Tris buffer containing 2 μM $ZnCl_2$ for 30 min to restore protein structure, gels were incubated at 50 °C for 30 min and then stained with 0.2% (w/v) toluidine blue O (T-3260, Sigma) in 10 mM Tris buffer for 10 min and destained twice for 20 min in 10 mM Tris buffer. With this method, the nuclease activity appears as a clear zone on a dark blue background.[11]

Cleavage of Glycan from ENDO2. To cleave the glycan moiety, the purified ENDO2 protein was digested with peptide *N*-glycosidase F (PNGase F) (170 μg/0.5 μg) in 10 mM Tris buffer, pH 7.0, at 37 °C for 24 h. The PNGase F-digested sample was analyzed with an 8−16% gradient SDS-PAGE. The glycoprotein was detected by a periodic acid Sciff (PAS) method on SDS-PAGE using a glycoprotein detection kit according to the manufacturer's instruction (Sigma). The enzymatic activity of ENDO2 (50 ng) treated with or without PNGase F was analyzed by in-gel ssDNase activity assay. To test whether the enzymatic activity was affected by protein refolding in SDS-washed PAGE, ENDO2 (0.1 μg) with or without *N*-glycan was incubated with 1 mg of ssDNA in 10 mM Tris buffer, pH 7.0, at 37 °C from 0 to 30 min. After digestion, samples were analyzed with 1.5% agarose gel.

Mass spectrometry analysis. The N-terminal sequence of the mature ENDO2 protein was identified using N-terminal Edman degradation by LC 492 Procise Protein Sequencing System (Applied Biosystems, Foster City, CA) at Technology Commons, College of Life Science in National Taiwan University. Peptides of ENDO2 digested with trypsin were analyzed using an LTQ mass spectrometer (MS) coupled with an online capillary liquid chromatography system [liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS)] (Thermo Fisher Scientific, MA) at the Proteomics Core Laboratory in Academia Sinica, Taiwan. The molecular weights of ENDO2 and PNGase F digested ENDO2 were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Bruker Daltonics, MA).

Circular Dichroism (CD) Spectroscopy. After gel filtration, the purified ENDO2 protein was digested with PNGase F in 10 mM Tris buffer, pH 7.0, at 37 °C for 24 h. ENDO2 and PNGase F-digested ENDO2 (20 μM, 250 μL) were placed in 1 mm path length cell maintained at room temperature. Protein CD spectra were recorded using a Jasco J-715 CD spectropolarimeter in the region of 195−250 nm (JASCO, United Kingdom).

Assay of ENDO2 Responses to Substrate Preference, Temperature, pH, Thermal Stability, and Cofactors. DNase and RNase activities were quantified according to the methods of Vogt[3] with minor modifications. Briefly, ENDO2 protein (0.1 μg) was incubated with 1 mg of ssDNA, dsDNA, or RNA substrate in buffer A (10 mM Tris buffer, pH 7.0, 2 μM $ZnCl_2$) at 37 °C from 0 to 180 min to monitor the dynamics of enzyme digestion. One fourth volume of cold 1 N HCl or 25% TCA was added and incubated on ice for 10 min to stop the reaction. After centrifugation at 16000*g* for 30 min, the supernatant was diluted with 20 mL of sterile deionized H_2O and measured at 260 nm to determine the amount of acid-soluble DNA or RNA. ENDO2 was incubated with 1 mg of ssDNA or RNA substrate

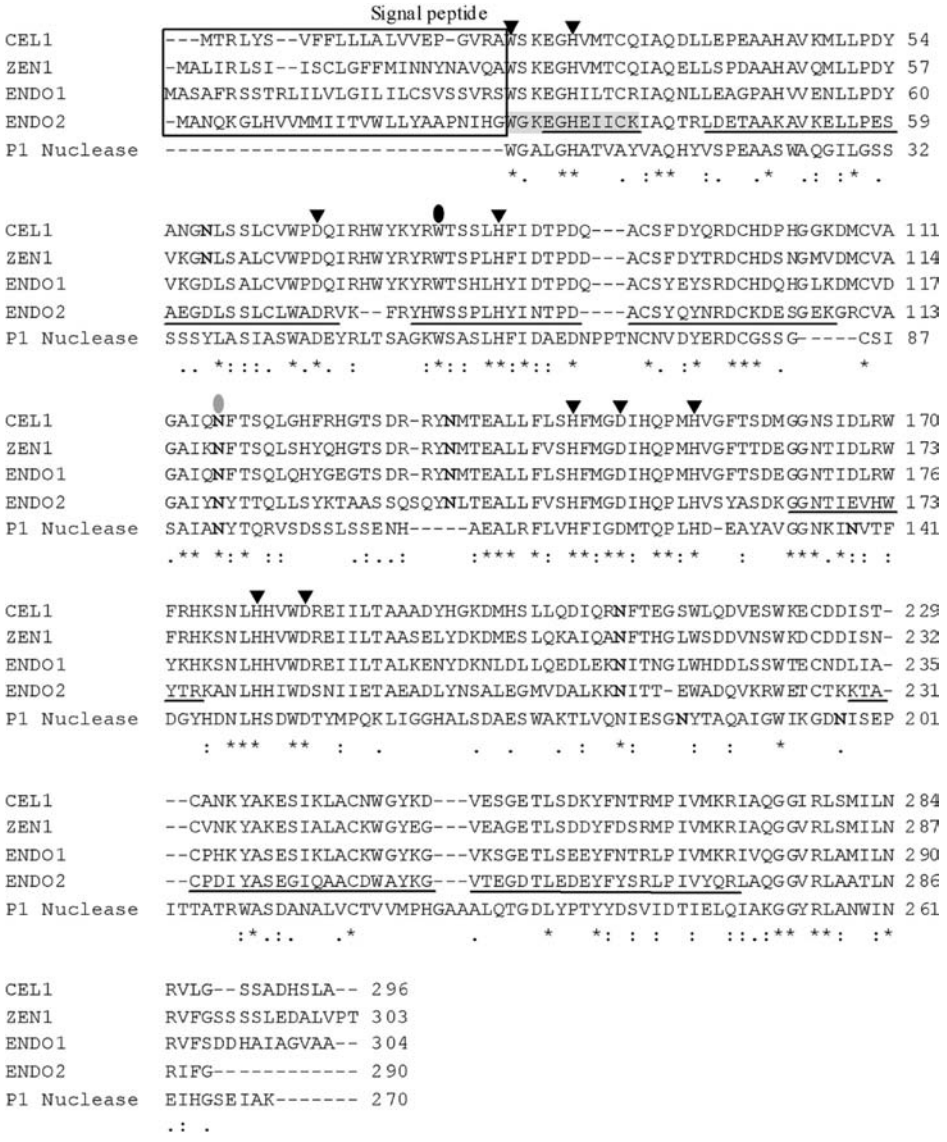

Figure 1. Alignment of ENDO1/2 homologues with P1 nuclease. Amino acid sequences of ENDO1 (At1g11190), ENDO2 (At1g68290), *A. graveolens* CEL1 (AAF42954), *P. citrinum* P1 nuclease (P24289), and *Z. elegans* ZEN1 (BAA28948) were aligned using the ClustalW program. Black triangles indicate residues that bind to zinc ions based on the structure of P1 nuclease, gray ellipse indicates the glycosylated site (Asn92) conserved in all nucleases, and black ellipse indicates the hydrophobic Trp55 residue. Boldfaced N indicates Asn residue predicted to be glycosylated. The signal peptides are indicated by a box. The N-terminal sequence of ENDO2 (shadowed in gray) was identified using N-terminal Edman degradation. Trypsin-digested ENDO2 protein was analyzed with LC-MS/MS, and detected peptides are underlined (44% coverage). Symbols "*", ":", and "." represent identity, strong similarity, and weak similarity.

in buffer A at temperatures from 10 to 90 °C for 10 min to measure the temperature dependence of its activity.

To examine the pH effect, ENDO2 (0.1 μg) was incubated with substrate (1 mg) in 100 μL of 30 mM Good's buffer (sodium acetate, cyclohexylaminopropanesulfonic acid, Bis-Tris propane, and bicine) with a pH range of 3−10 at 37 °C for 10 min.

To scrutinize thermal stability, ENDO2 was preheated at various temperatures from 60 to 90 °C in buffer A for 10 min. After heat treatment, the samples were quickly cooled, and the remaining enzyme activities were analyzed. One unit (U) of ENDO2 activity is defined as 1 μmol of acid soluble nucleotides produced from RNA, ssDNA, or dsDNA per min. The specific enzyme activity was calculated as U/mg = μmol min^{-1} mg^{-1}.

To analyze the effect of cofactors, ENDO2 (0.1 μg) was incubated in 10 mM Tris buffer, pH 7.0, with 1 mg of ssDNA or RNA containing each of the different cofactors including 0.002 mM $ZnCl_2$, 1 mM $ZnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 100 mM NaCl at 37 °C for 10 min.

Comparison of ENDO2 and P1 Nuclease Activity. The kinetic parameters of ENDO2 were examined by measuring enzyme activities at various concentrations (0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 mg mL^{-1}) at 37 °C for 20 min using ssDNA substrate. The K_m and V_{max} values of ENDO2 were calculated with the Michaelis−Menten method.

To compare enzymatic activities, ENDO2 and P1 nuclease (0.1 μg each) were incubated with 1 mg of ssDNA, dsDNA, or RNA in buffer A and buffer B (30 mM acetate buffer, pH 5.3, 1 mM $ZnCl_2$, and 50 mM NaCl) at 37 °C for 10 min. P1 nuclease was purchased from Sigma (N8630), and buffer B is recommended for its digestion.

Mismatch Detection Assay. GFP gene was used as template for two rounds of PCR amplification with 1, 2, 3, or 6 bases inserted at position 210 using degenerate mutagenic primer sets (Table S1 in the Supporting Information) with a GeneAmp PCR System 2400 (Applied Biosystems) as previously described.[29] The PCR products were ligated into pGEM-T easy vector and confirmed with sequencing. The mismatches were created by mixing equal quantities of the PCR products to prepare heteroduplex DNA. Aliquots of 1.5 μg of PCR

products were heated to 99 °C for 10 min, followed by 90 cycles with the temperature decreased by 0.3 °C per cycle, and renatured at 70 °C for 60 s.[30] The heteroduplex templates were incubated with 2 ng of ENDO2 at 37 °C for 40 min in 20 μL of CEL1 digestion buffer [10 mM HEPS buffer, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 0.002% Triton X-100, and 0.0002 mg mL^{-1} bovine serum albumin (BSA)]. Reactions were terminated by adding 5 μL of 150 mM EDTA. The digested heteroduplex products were analyzed with 1.5% agarose gel.

RESULTS

Characterization of the ENDO2 Gene Product. The *ENDO2* open reading frame contains 873 bases encoding 290 amino acids. The predicted molecular mass of the mature protein without the 27 residue signal peptide is 29.7 kDa. Amino acid sequences of *Arabidopsis* ENDO1 (At1g11190), ENDO2 (At1g68290), *A. graveolens* CEL1 (AAF42954), *Z. elegans* ZEN1, and *P. citrinum* P1 nuclease (P24289) were aligned using the ClustalW program (Figure 1). Three N-glycosylated sites at positions 118, 137, and 211 (91, 110, and 184 of mature protein) in ENDO2 are predicted based on the consensus sequence of Asn-X-Ser/Thr, suggesting that ENDO2 is a glycoprotein. The Asn residue (92 in P1 nuclease, 118 in ENDO2), which is found to be glycosylated in P1 nuclease, is conserved in all nucleases (Figure 1). According to the structure of P1 nuclease, Asn118 in ENDO2 (Asn92 in P1 nuclease) may be glycosylated and form a sugar hydrophilic pocket to prevent the exposure of Trp80 (Trp55 in P1 nuclease) and to maintain structural stability.[9,10]

The recombinant ENDO2 protein was extracted from *Arabidopsis* transgenic plants. Trypsin-digested ENDO2 peptides were analyzed with LC-MS/MS in combination with Edman degradation. Our results confirmed that the N-terminal sequence of mature ENDO2 is WGKEGHEII and the first 27 amino acids were removed from the mature protein (Figure 1). Residues Trp28, His33, Asp72, His85, His147, Asp151, His157, His181, and Asp185 in ENDO2 are identical to those in P1 nuclease, which are for zinc ion coordination (Figure 1).[7,11,13]

ENDO1/2 Produced in Transgenic *Arabidopsis* Plants May Be Post-translationally Modified. The *Arabidopsis* ENDO1 and ENDO2 mature proteins were expressed in *E. coli* IMPACT and pMAL system separately; however, these ENDO proteins did not exhibit nuclease activity, perhaps because of the lack of glycosylation and improper folding. Additionally, overexpression of the ENDO1 and ENDO2 in *P. pastoris* with pPICZαC and pPIC9K vectors did not secrete any ENDO protein. Therefore, the *ENDO1/2* cDNA was cloned into pBI121 to form 35SP::ENDO1/2-HisOE (Figure 2A) and overexpressed in *A. thaliana*. The protein extracted from transgenic plants carrying 35SP::ENDO1/2-HisOE showed DNase (Figure 2B) and RNase (Figure 2C) activities using ssDNA and RNA as substrates. ENDO2 exhibited a higher activity than ENDO1 and S-like ribonuclease (RNS) activity in the RNase assay gel (Figure 2C). Protein extracted from transgenic line 6 of ENDO2 displayed the highest nuclease activity; thus, the offspring of line 6 was used for further experiments.

Chlorophyll in the crude protein extract interfered strongly with the purification of ENDO2; hence, a DEAE column was used in the first step of purification. The negatively charged chlorophyll binds to this anion exchange resin tightly and allows ENDO2 to be eluted with 250 mM NaCl and detected by SDS-PAGE. The collected ENDO2 fractions were applied to a nickel column, then eluted with imidazole (Figure 3A), and

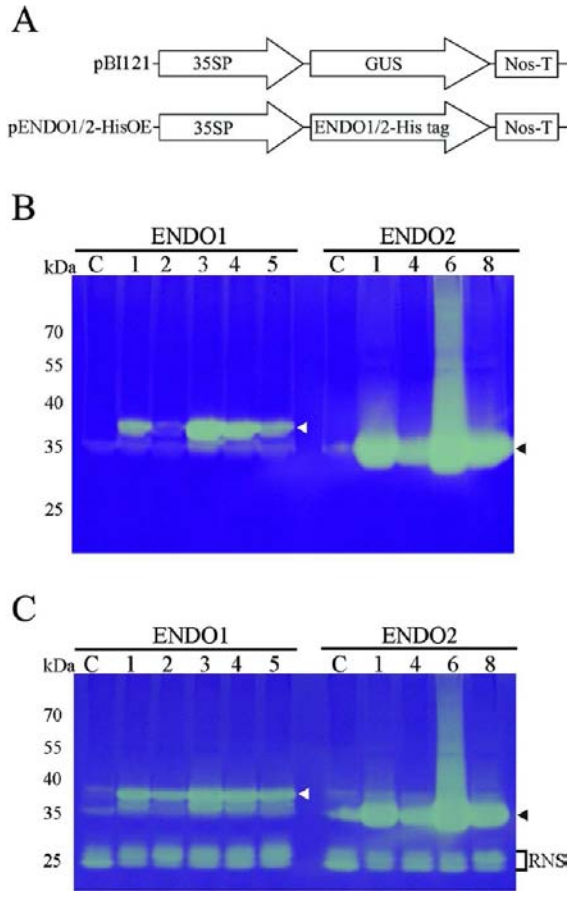

Figure 2. ENDO proteins overexpressed in transgenic *Arabidopsis* plants. (A) Schematic representation of pENDO1-HisOE and pENDO2-HisOE constructions. ENDO1 and ENDO2 cDNA were cloned into pBI121 vector between the CaMV 35S promoter (35SP) and the transcriptional terminator (Nos-T). Control plants were transformed with the pBI121 binary vector and designated C. (B) DNase activity gels of ENDO1 and ENDO2. Each sample of 50 μg of total protein from transgenic lines, respectively, was loaded in DNase activity assay gel with 0.8 mg mL^{-1} salmon ssDNA. (C) The RNase activity of ENDO1 and ENDO2 was assayed with 2.4 mg mL^{-1} yeast RNA. Enzyme activities that degrade the RNA or ssDNA substrate in the gel appear as clear bands on a dark background. The protein size standards are indicated. White and black arrow heads show the ENDO1 and ENDO2 activity, respectively. RNS indicates ribonucleases.

confirmed by Western blot (Figure 3B). Coomassie staining of the 12% SDS-PAGE displayed a significant band (MW ~34 kDa) in the 100 mM imidazole fraction (Figure 3A, lane 5). A yield of 2.6 mg of ENDO2 enzyme was obtained from 50 g of 2 week seedlings (Table 1). Monoclonal anti-His tag antibody detected the His-tagged proteins ENDO2 (Figure 3D, lane 4) and VrDhn1 (Figure 3D, lane 2) but not BSA or P1 nuclease (Figure 3C, lanes 1 and 3). DNase (Figure 3E) and RNase (Figure 3F) assay gels were run in parallel to confirm the activities of ENDO2 (lane 4) and P1 nuclease (lane 3). In comparison to the predicted mature ENDO2 with 6 × His tag (30.55 kDa), the higher molecular mass of the purified protein could be attributed to N-linked glycoprotein at positions 118, 137, and/or 211. Our results indicated that ENDO2 may be an N-glycosylated protein.

N-Glycosylation Is Requisite for ENDO2. ENDO2 has three putative N-glycosylation sites at Asn118, Asn137, and

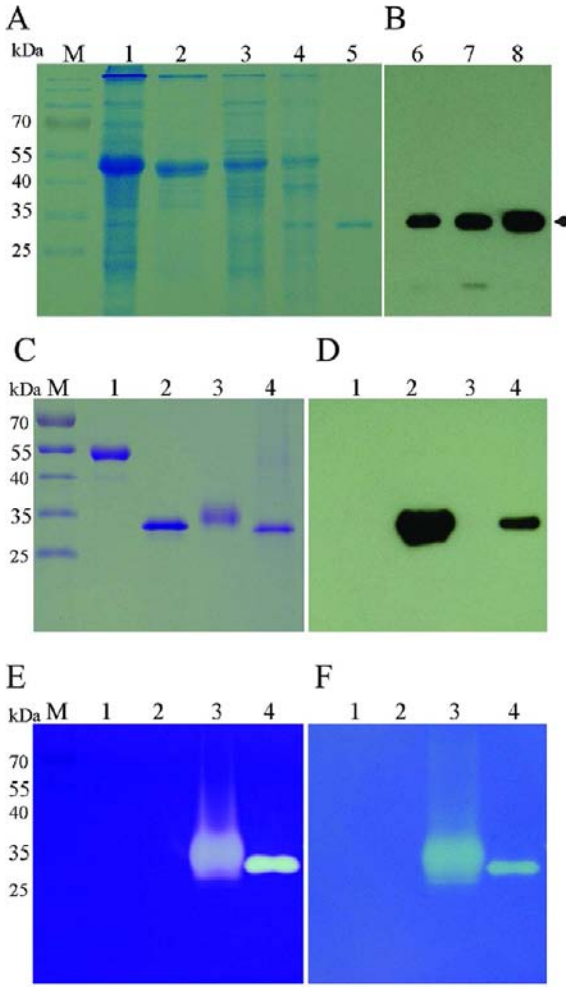

Figure 3. Purification of ENDO2 protein from transgenic plants. (A) Different fractions of ENDO2 protein collected from the nickel column. M indicates protein markers; the lane 1 sample was a collected fraction of 250 mM NaCl from DEAE column; lane 2 was a flow-through solution; and lanes 3–5 were eluted with 25, 50, and 100 mM imidazole. Each lane contained 10 μL of sample. (B) The imidazole elutions (lanes 3–5 in A) were transferred to PVDF membrane (lanes 6–8) and hybridized with monoclonal anti-His tag antibody. The arrow head indicates the ENDO2. (C) SDS-PAGE of ENDO2-His tag protein (lane 4). M indicates protein markers, lane 1 is BSA, lane 2 is a VrDhn1-His tag protein used as a negative control, and lane 3 is P1 nuclease. (D) Western blot was performed using a monoclonal anti-His tag antibody. Lanes 1–4 contained the same samples as lanes 1–4 in C. DNase (E) and RNase (F) activities were analyzed with in-gel assay using the samples listed in C. Each lane contained 50 ng of sample.

Asn211 (91, 110, and 184 of mature protein). The amino acid sequence of mature ENDO2 with 6× His tag is calculated as 30.55 kDa. ENDO2 in transgenic plants has a molecular mass of 34.5 kDa (Figure 4A, lane 2), showing that the protein has been modified. Application of PNGase F to ENDO2 cleaved the innermost GlcNAc from Asn of N-glycosylated protein and resulted in two bands (~31.8 kDa and 30.5 kDa) in the gel (Figure 4A, lane 3). To further confirm that ENDO2 is a glycoprotein, the gel was stained with PAS method to detect oligosaccharides as magenta bands in light pink background. PNGase F digestion of ENDO2 resulted in a 31.8 kDa protein as a light magenta color band, but the 30.5 kDa protein cannot be detected (Figure 4B, lane 3). It is possible that the 31.8 kDa

Table 1. Purification of ENDO2 from a 50 g *A. thaliana* Transgenic Plant

purification step	volume (mL)	total protein (mg)	enzymatic activity[a] (U)	specific activity (U/mg)	fold purification[b]
original buffer	162.0	542.3	2283.8	4.2	1.0
DEAE column	95.5	122.6	1749.9	14.3	3.4
nickel column	10.0	2.6	940.3	361.7	85.9

[a]The ssDNase activity was measured in 10 mM Tris buffer, pH 7.0, containing 2 μM $ZnCl_2$ at 37 °C. [b]The fold purification was calculated based on specificity.

protein still carried glycans and thus can be detected with PAS staining. All of the glycans can be removed from ENDO2 to form the 30.5 kDa band, which cannot be detected by PAS staining. The exact molecular masses of ENDO2 and the PNGase F digested peptides were determined by MALDI-TOF MS. Our data showed that ENDO2 has a molecular mass of 34.527 kDa (Figure 4F), and the two PNGase F digested bands are 31.821 and 30.548 kDa (Figure 4G). A protein of 17.589 kDa contaminated in our purified ENDO2, which was confirmed by LC-MS/MS as a putative polyketide cyclase/dehydrase encoded by the At4g23670 gene.

N-Glycan was released from ENDO2 to investigate the effect of N-glycosylation in enzyme activity. The removal of *N*-glycan diminished ENDO2 endonuclease activity (Figure 4C, lanes 2 and 3; Figure S1 in the Supporting Information, right panel). To examine whether the removal of sugar groups from the protein affects the enzyme stability or activity, the stability of the protein was analyzed before and after deglycosylation using CD spectropolarimeter. ENDO2 contains mostly α-helix (84%); however, the removal of *N*-glycan from ENDO2 reduced α-helical content and the stability of secondary structure (Figure 4H). Our results validated that ENDO2 enzyme stability and function is modulated by N-glycosylation.

Enzymatic Properties of ENDO2. The ENDO2 enzyme digested three different substrates at 37 °C with preference of ssDNA > RNA > dsDNA (Figure 5A). The ssDNA was completed digested by ENDO2 after 40 min. ENDO2 hydrolyzed 17.2-fold of ssDNA (36.19 μg min^{-1}) and 3.6-fold of RNA (7.48 μg min^{-1}) as compared to dsDNA (2.10 μg min^{-1} set as 1) at pH 7.0 for 20 min. Neither the RNase nor the dsDNase activities reached a plateau within 3 h. To test the optimal temperature of ENDO2, reactions were performed with ssDNA and RNA from 20 to 90 °C. The hydrolytic activity of ENDO2 was low at temperatures below 30 °C and reached a maximum at 70 °C (Figure 5B). A 10 °C increase in temperatures between 40 and 60 °C approximately doubled hydrolysis of substrates catalyzed by ENDO2. Above 70 °C, the ENDO2 catalytic activity declined. While ENDO2 ssDNA hydrolysis exhibited a neutral pH optimum, RNA hydrolysis showed an optimum at pH 6 (Figure 5C). The RNA substrate is unstable at pH above 8.0; thus, RNA hydrolytic activity was measured below pH 8. To test the heat stability of ENDO2, reactions were performed with ssDNA and RNA from 60 to 90 °C after preheating the enzyme for 10 min. The catalytic activity of ENDO2 declined above 62.5 °C (Figure 5D), indicating that ENDO2 is a heat stable ENDO.

The ENDO2 ssDNase activity was not significantly affected in the presence of 2 μM Zn^{2+} cofactor but was decreased 65%

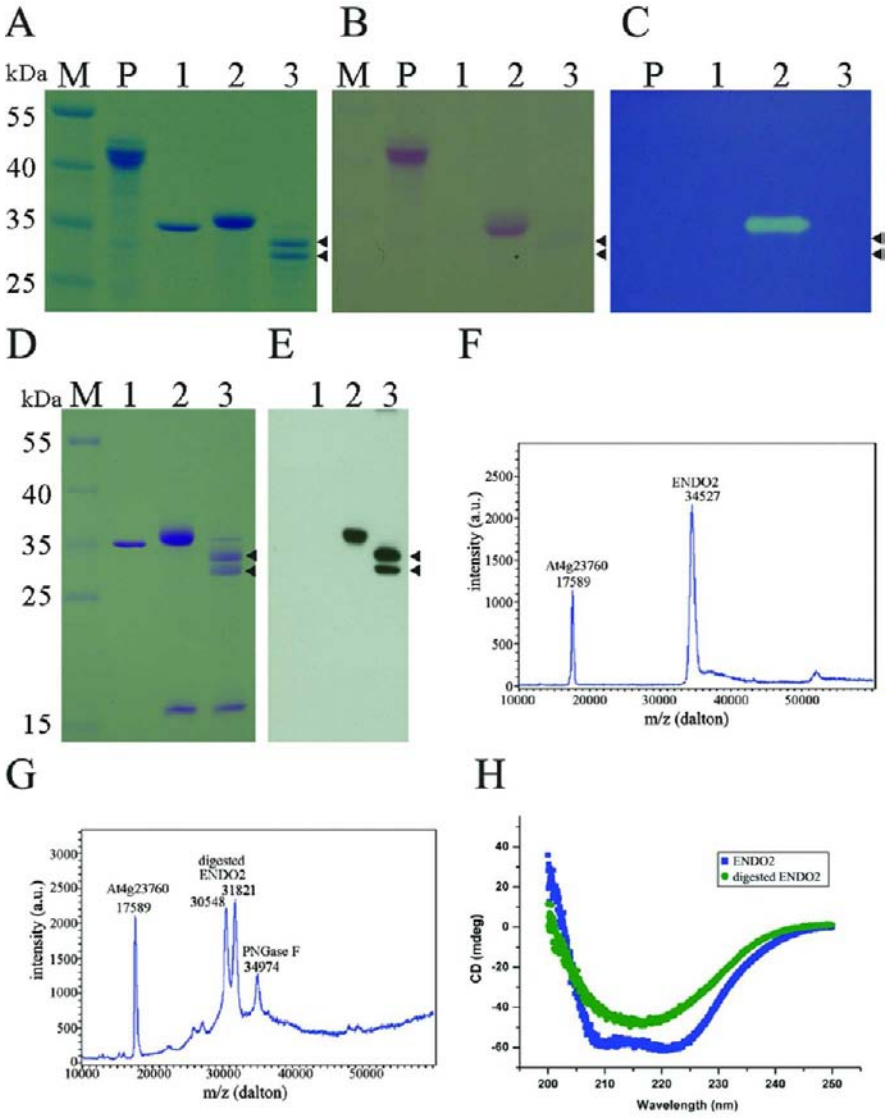

Figure 4. Effects of N-glycan on the function of ENDO2. (A–C) ENDO2 (5 μg) was treated with (lane 3) or without (lanes 2) PNGase F in 10 mM Tris buffer, pH 7.0, at 37 °C for 24 h. (A) Coomassie blue staining SDS-PAGE, (B) PAS staining for glycan, and (C) in-gel ssDNase activity assay. M indicates protein markers, P is peroxidase as a positive control of glycoprotein, and lane 1 is PNGase F protein. Arrow heads indicate the two bands of ENDO2 after PNGase F digestion. ENDO2-His tag proteins digested with (lane 3) or without (lanes 2) PNGase F were resolved in SDS-PAGE and stained with Coomassie blue (D), and the proteins were detected with monoclonal anti-His tag antibody (E). The molecular mass of ENDO2 (F) and PNGase F digested ENDO2 (G) were determined by MALDI-TOF MS. The secondary structures of ENDO2 and PNGase F digested ENDO2 were measured with a CD spectropolarimeter (H).

by 1 mM Zn^{2+} (Figure 6A). The presence of 1 mM Ca^{2+} or Mg^{2+} had no effect on ENDO2 ssDNase activity. The addition of the divalent cation chelator (EDTA) and reducing agent (DTT) both decreased ENDO2 ssDNase activity ($P < 0.01$). Although 50 mM NaCl did not affect ENDO2 ssDNase activity, an increase of concentration to 100 mM caused a 20% decrease in ssDNase activity.

Divalent metal ions significantly decreased ENDO2 RNase activity (Figure 6B). The addition of 1 mM Ca^{2+} or Mg^{2+} or 2 μM Zn^{2+} decreased 40% of ENDO2 RNase activity, and 1 mM Zn^{2+} decreased 70% activity. Interestingly, the chelator EDTA enhanced ENDO2 RNase activity 35% (Figure 6B) but decreased ssDNase activity (Figure 6A). The RNase activity of ENDO2 was not significantly affected by NaCl. The addition of 1 mM DTT, which may have cleaved the disulfide bond of ENDO2, also caused a 40% decrease in RNase activity. Our data showed that the exogenous Ca^{2+} or Mg^{2+} inhibited RNase activity but not ssDNase activity of ENDO2.

Comparison of Enzymatic Activities of ENDO2 and P1 Nuclease. ENDO2 enzyme displayed a higher activity than P1 nuclease for all three substrates. ENDO2 had a higher catalytic efficiency in buffer A (10 mM Tris buffer, pH 7.0, 2 μM $ZnCl_2$) than buffer B (30 mM acetate buffer, pH 5.3, 1 mM $ZnCl_2$, and 50 mM NaCl). Buffer B is the digestion buffer for P1 nuclease according to the manufacturer's protocol. Thus, both buffers were used to compare the substrate catalytic activities of ENDO2 and P1 nuclease. ENDO2 showed higher ssDNase activity in buffer A (361.7 ± 17.2 U mg^{-1}) than in buffer B (223.2 ± 10.4 U mg^{-1}) (Figure 7A). P1 nuclease exhibited much lower ssDNase activity ($P < 0.01$) in either buffer. The ENDO2 RNase activity was similar to P1 nuclease (Figure 7B). The ENDO2 dsDNase activity was lower in buffer A (14.1 ±

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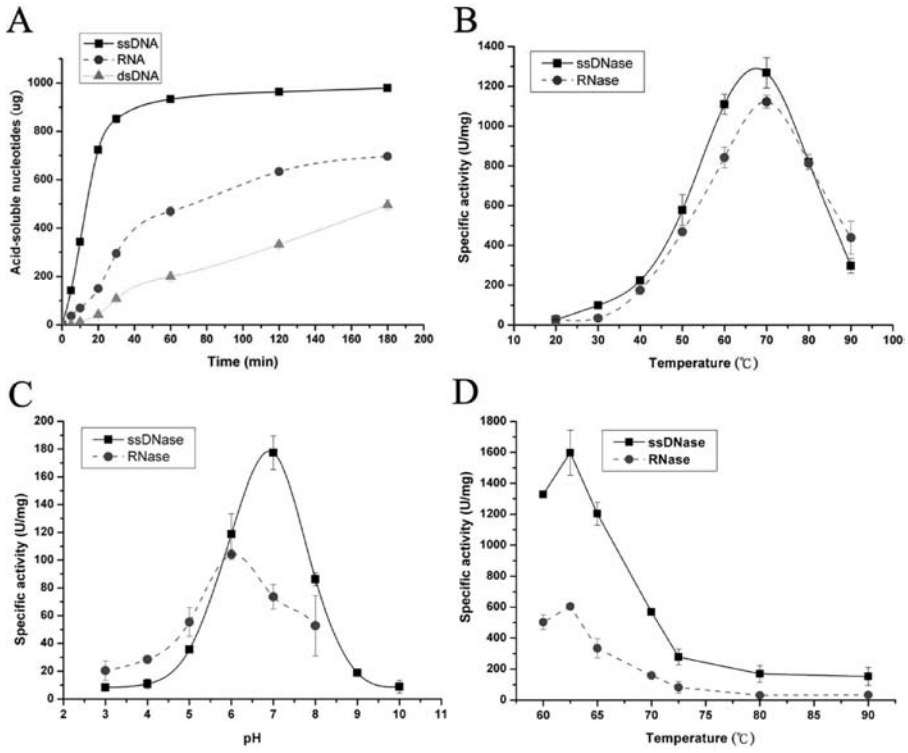

Figure 5. Characterization of the ENDO2 protein. (A) Substrate specificity was assayed by incubation of ENDO2 protein in 10 mM Tris buffer, pH 7.0, with 2 μM $ZnCl_2$ in the presence of 1 mg of ssDNA (square), 1 mg of RNA (circle), or 1 mg of dsDNA (triangle) at 37 °C for different times. (B) The optimal temperature of ENDO2 protein was determined by reactions at different temperatures for 10 min. (C) The optimal pH of ENDO2 enzymatic activity was measured in pH 3−10 of 30 mM Good's buffer at 37 °C for 10 min. (D) The thermal stability of ENDO2 was measured at various temperatures in buffer A for 10 min after preheating the enzyme for 10 min. Data represent the mean ± SD of three independent experiments.

0.9 U mg^{-1}) than in buffer B (45.9 ± 8.9 U mg^{-1}) (Figure 7A,C). Both ENDO2 and P1 nuclease displayed higher dsDNase activity in buffer B, while ENDO2 catalysis was 10-fold of P1 nuclease ($P < 0.01$) (Figure 7C).

Kinetic analysis of ENDO2 and P1 nuclease was performed using ssDNA as substrate at 37 °C in 10 mM Tris buffer containing 2 μM Zn^{2+} (buffer A) or containing 1 mM Zn^{2+}. To determine the Michaelis constants (K_m) and catalytic activity (k_{cat}) values of ENDO2 and P1 nuclease, nuclease assays were carried out using various concentrations of ssDNA substrate and a constant amount of enzyme, and the initial cleavage rates were measured by quantifying cleavage products. The K_m values derived from these experiments in buffer A were 8.60 ± 0.35 and 16.62 ± 0.41 mM, and k_{cat} values were 19171 ± 319 and 27784 ± 442 min^{-1} for ENDO2 and P1 nuclease, respectively. A comparison of kinetic parameters for ENDO2 and P1 nuclease is shown in Table 2. The lower K_m value and higher k_{cat}/K_m indicates that ENDO2 has a higher binding affinity to ssDNA than P1 nuclease, leading to the higher catalytic efficiency of ENDO2.

ENDO2 Recognizes and Cleaves Mismatches in DNA. To examine whether ENDO2 recognizes mismatches in DNA, we designed a set of plasmids containing different base insertions at 210−211 bp position of the GFP gene (720 bp). The PCR products were combined to produce all types of mismatches in heteroduplex DNA (Table S2 in the Supporting Information). After denaturation and renaturation, heteroduplex DNA and homoduplex DNA in a ratio of 1:1 were formed for identifying single base pair mismatches in heteroduplex DNA (Figure S2 in the Supporting Information). For instance, pooled two identical PCR reactions containing the same insertion at 211 bp position (X_1 = A and X_2 = A, indicated as A in Figure 8B), homoduplex DNA, and heteroduplex DNA formed in a ratio of 1:1, and the heteroduplex DNA contains no mismatch. In a pool of two PCR reactions containing different insertions at 211 bp position (X_1 = A and X_2 = G, indicated as A+G in Figure 8B), the heteroduplex DNA contained two types of single base pair mismatches (A/C and G/T). ENDO2 was found to recognize and cleave all types of single base pair mismatches to produce two DNA fragments of 210 and 510 bp on agarose gel (Figure 8, lanes 7−12). Our data also showed that ENDO2 cleaves at bulge loops of 1, 2, 3, and 6 nucleotides mismatches in heteroduplex DNA (Figure 9A, lanes 5−8). Similarly, heteroduplex DNA, which carried a loop on both strands, was recognized and cleaved by ENDO2 to generate two DNA fragments (Figure 9B, lanes 5−8).

CEL1 unwinds at an A + T-rich region in homoduplex DNA and results in nonspecific DNA degradation of the A + T-rich region.[12] The mismatch-cleavage activity of ENDO2 was compared with ENDO1 and CEL1, and all of the three nucleases exhibited a mismatch-cleavage activity (Figure 10). Similar to CEL1, ENDO1 or ENDO2 unwinds at an A + T-rich region, resulting in nonspecific DNA degradation. All of the three ENDOs showed a high mismatch-cleavage activity and digested heteroduplex DNA containing single base pair or multibase mismatches more efficiently than homoduplex DNA (Figure 10).

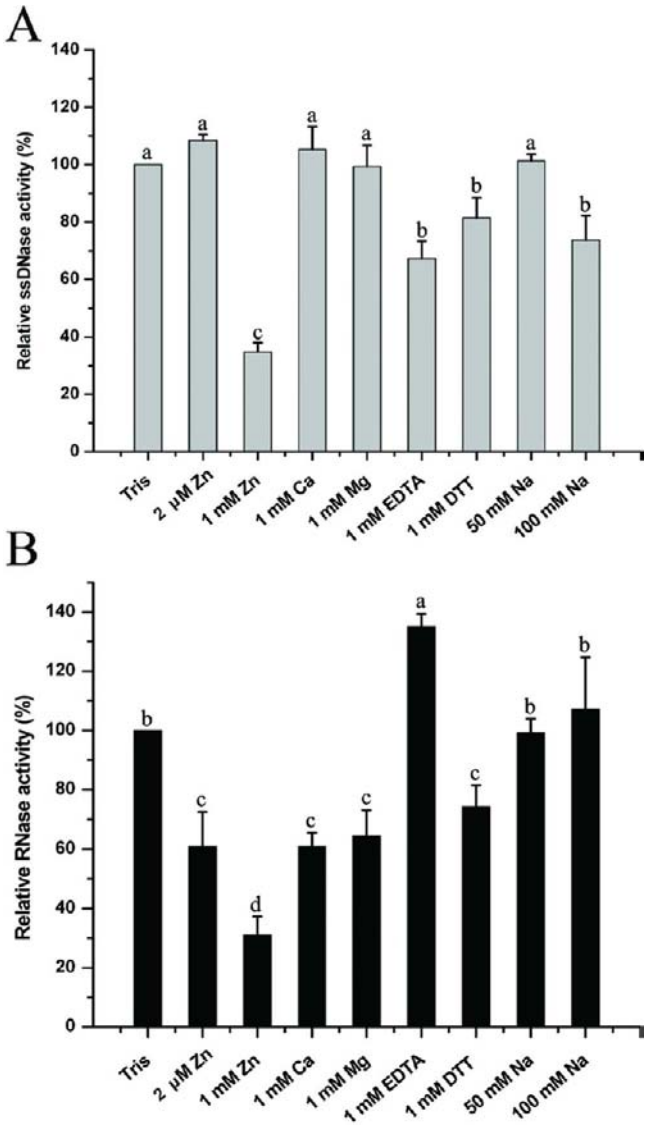

Figure 6. Effects of cofactors on the ENDO2 protein activity. ENDO2 ssDNase activity (A) and RNase activity (B) were measured in 10 mM Tris buffer, pH 7.0, with 1 mg of ssDNA or RNA containing different cofactors as indicated. Tris represents Tris buffer only. Data represent the mean ± SD of three independent experiments. Values in each column followed by different letters are significantly different (P < 0.01) according to one-way ANOVA test.

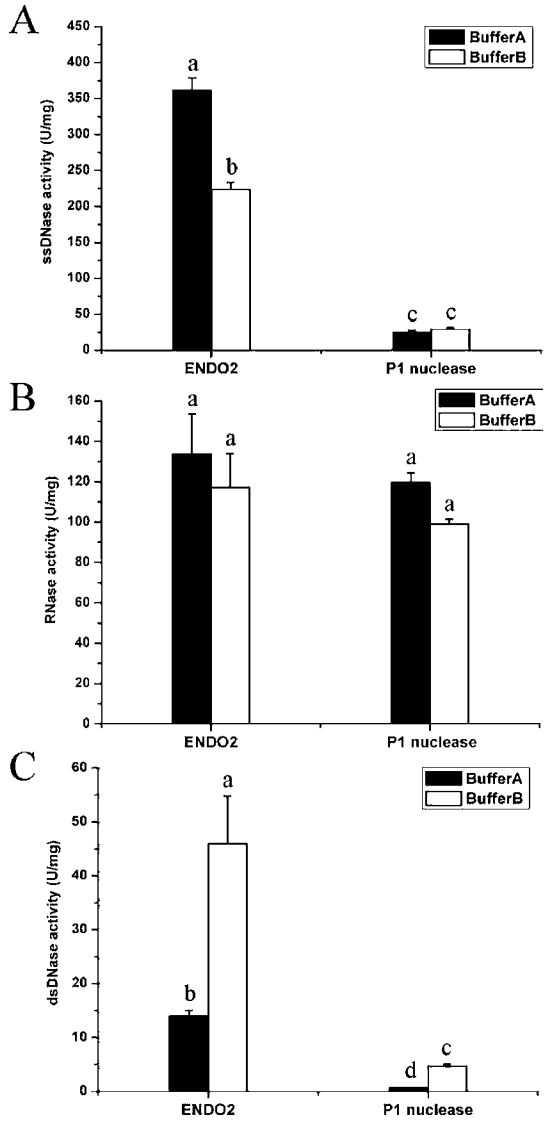

Figure 7. Comparison of enzyme activities between ENDO2 and P1 nuclease. ENDO2 and P1 nuclease proteins were incubated with 1 mg of ssDNA (A) or RNA (B) or dsDNA (C) at 37 °C in buffer A (solid column; 10 mM Tris, pH 7.0, 2 μM $ZnCl_2$) and buffer B (open column; 30 mM acetate buffer, pH 5.3, 1 mM $ZnCl_2$, and 50 mM NaCl) at 37 °C for 10 min. Data represent the mean ± SD of three independent experiments. Values in each column followed by different letters are significantly different (P < 0.01) according to two-way ANOVA test.

DISCUSSION

ENDO2 was produced by overexpressing At1g68290 gene in *Arabidopsis* plants since the protein could not be efficiently produced with *E. coli* or yeast expression systems. The plant ENDO2, ENDO1,[11,13] CEL1,[12,14] and ZEN1[20] peptides share highly conserved residues in the active site for catalysis and the N-glycosylation site (Asn92) for protein stability with P1 nuclease (Figure 1).[9,10] N-Glycosylation of eukaryotic proteins helps folding and traverse the cellular secretory pathway, and ENDO2 produced in *E. coli* lost function may be due to the lack of *N*-glycan. Differences between N-glycosylation in plant and yeast may cause the failure of yeast system in ENDO2 production. Digestion of ENDO2 (34.527 kDa, ~22 sugars) with PNGase F resulted in the peptides of 31.821 (~7 sugars) and 30.548 kDa (no sugar). Removal of *N*-glycan (~15 sugars) from one or two sites produced the 31.813 kDa peptide, which showed destabilized structure and lost enzymatic activity (Figure 4). Asn118 is the putative glysosylation site based on the structure of P1 nuclease. *N*-Glycan has been reported to be required for full enzymatic activity of human fucosyltransferase III[31] and galactosylceraminde sulphotransferase.[32] Our data validated that glycosylation is important to maintain ENDO2 stability, and removal of *N*-glycan destabilized the protein and diminished enzymatic activity. To our knowledge, this is the first report verifying the significance of N-glycosylation in the enzymatic function of plant ENDOs.

Although ENDO2 is a zinc-dependent enzyme, ENDO2 ssDNase and RNase activity was marked decreased by adding 1 mM zinc ions (Figure 6). This inhibition could be resulted from the high molar ratio of zinc ion to ENDO2. The ENDO2 RNase activity was reduced by all of the examined divalent

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Table 2. Kinetic Analysis of ENDO2 and P1 Nuclease ssDNase Activity[a]

zinc concn in reaction buffer	parameter	ENDO2	P1 nuclease
2 μM (buffer A)	k_{cat} (min^{-1})	19171 ± 319	27784 ± 442
	K_m (mM)	8.60 ± 0.35	16.62 ± 0.41
	k_{cat}/K_m	2230 ± 56	1672 ± 14
1 mM	k_{cat} (min^{-1})	7636 ± 259	29276 ± 334
	K_m (mM)	8.34 ± 0.76	19.00 ± 0.61
	k_{cat}/K_m	919 ± 63	1542 ± 33

[a]The kinetic parameters were calculated by the Michaelis−Menten equation with nonlinear regression. The experiments were carried out using ssDNA as substrate at different concentrations in 10 mM Tris buffer, pH 7.0, at 37 °C with Zn^{2+} ion at 2 μM or 1 mM. Values are means ± SDs of three independent experiments.

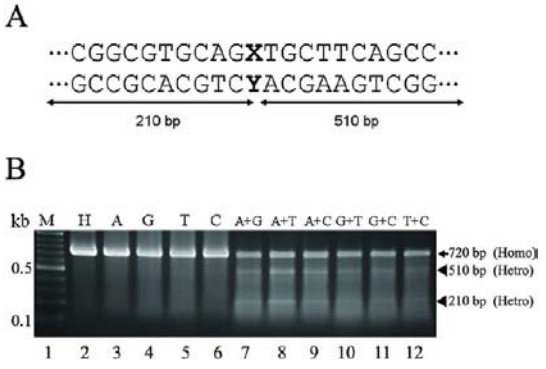

Figure 8. ENDO2 recognizes and cleaves base substitution mismatches. (A) Schematic representation of the homo- and heteroduplexes containing base substitutions amplified by PCR. **X** and **Y** represent the variations of each base (**X** = A, G, T, or C) and its complementary base (**Y** = T, C, A, or G). (B) PCR products were digested with ENDO2 and analyzed with 1.5% agarose gel. Each lane contains two sets of PCR products, which were denatured and reannealed. Reannealing of PCR products with the same base created homoduplex DNA (lanes 3−6). Combinations of a group of four nucleotides taken two at a time is C(4,2) = 6 (lanes 7−12); thus, two sets of PCR products with a single base difference created one-half heteroduplex DNA (lanes 7−12). H represents the homoduplex DNA of GFP (lane 2). Lane 1 is a DNA marker.

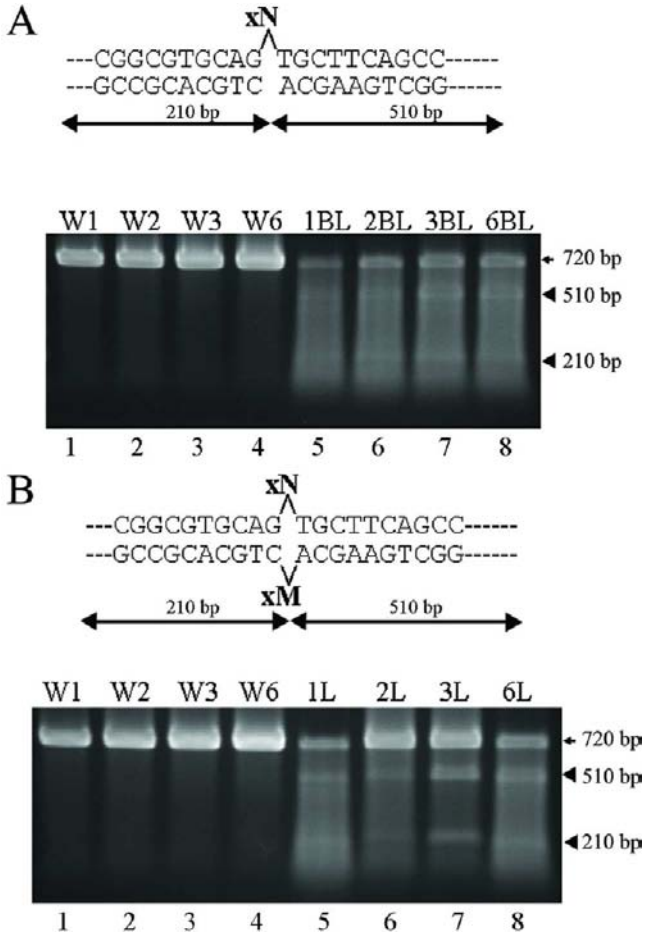

Figure 9. ENDO2 recognizes and cleaves heteroduplex DNA containing bulge loops [(BL), A] and loops [(L), B]. N and M represent each of the four types of nucleotides, and *x* represents 1, 2, 3, or 6 bp insertions. W1, W2, W3, and W6 (lanes 1−4) indicate wild-type dsDNA with 1, 2, 3, or 6 nucleotides insertion as homoduplex DNA, while xBL and xL indicate dsDNA with 1, 2, 3, or 6 nucleotides insertion as heteroduplex DNA (lanes 5−8). ENDO2 digestion products (triangles) were analyzed with 1.5% agarose gel.

cations, and the reduction was reversed by the addition of EDTA. ENDO2 may possess different binding sites for DNA and RNA, EDTA reduced the concentration of divalent cations, and RNase does not require divalent cations for activity. Similarly, it was reported that the ssDNase but not RNase of the chloroplast ENDO from wheat is strongly inhibited by EDTA.[33] Recently, an EDTA-dependent RNase activity causing RNA degradation in *Arabidopsis* suspension culture was reported.[34] According to the *Arabidopsis* microarray data available on the Genevestigator Web site,[35] ENDO2 is highly expressed in suspension culture.

ENDO2 has a similar thermal stability and optimum temperature with P1 nuclease, but the pH optimum range of P1 nuclease (pH 5−8) is relatively broad. ENDO2 has a higher ssDNase activity than P1 nuclease. In addition, ENDO2 possesses the ability to cleave single base pair mismatches in heteroduplex DNA (Figure 8), while P1 nuclease cleaves loops with more than three base pair mismatches.[36] ENDO2 has a relatively lower Michaelis constant than P1 nuclease, which may contribute to its higher catalytic efficiency.

The plant expression system offers benefits for eukaryotic protein expression, including correct protein structure, disulfide bond formation, and post-translational modification. For instance, active recombinant human Flt3 ligand was expressed and purified from transgenic barley seeds.[37] Chicken secretory antibodies can be produced using *Nicotina benthamiana* leaves.[38] Multiple genes designed for β-carotene synthesis were transformed to rice to produce provitamin A.[39] Plants green factories can produce proteins, unless the candidate protein is lethal or causes sterility in transgenic plants. Our results showed that the homologous transgenic system carrying 35SP::ENDO2 stably produced ENDO2 enzyme at a high level without affecting plant growth. Perhaps ENDO2 enzyme is confined to a specific organelle, and this possibility is worth further investigation. Purification of 50 g transgenic plants produced 2.6 mg of ENDO2 enzyme, which can be used for 10 million mismatch detections. In comparison, celery stalks of 105 kg produced 0.005 mg of CEL1 enzyme.[14] Recombinant protein production using transgenic plants as bioreactors is a good strategy to continuously produce large amounts of protein at low cost.

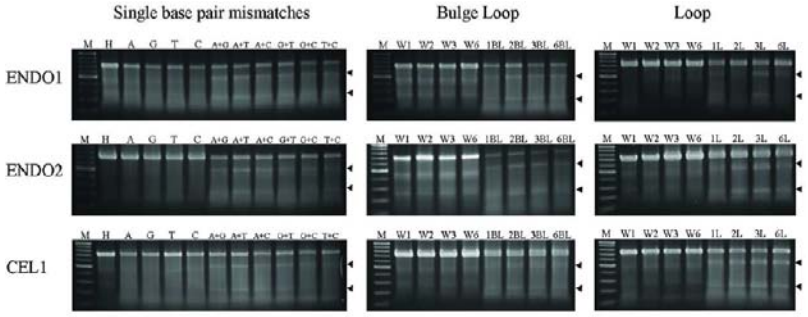

Figure 10. Comparison of mismatch cleavage activity in ENDO1, ENDO2, and CEL1. W represents the wild-type dsDNA of GFP, and W1, W2, W3, and W6 indicate wild-type dsDNA with 1, 2, 3, or 6 nucleotides insertion as homoduplex DNA. ENDO2 digestion products (triangles) were analyzed with 1.5% agarose gel.

■ ASSOCIATED CONTENT

Supporting Information

Primers used for PCR amplification of constructs applied in this study (Table S1), summary of heteroduplex DNA formed from mutation pairs (Table S2), the activity of ENDO2 and PNGase F digested ENDO2 (Figure S1), and schematic representation of homoduplexes and heteroduplexes formed by the combination of two PCR-amplified products (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +886 3 5742758. Fax: +886 3 5715934. E-mail: tylin@life.nthu.edu.tw (T.-Y.L.). Tel: +886 2 27871043. Fax: +886 2 27827954. E-mail: boplshaw@gate.sinica.edu.tw. (J.-F.S.).

Present Address

#Biodiversity Research Center, Academia Sinica, Taipei 11529, Taiwan.

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Notes

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■ ABBREVIATIONS USED

dsDNA, double-stranded DNA; EMS, ethyl methane sulfonate; ENDO, endonuclease; MNU, *N*-methyl-*N*-nitrosourea; LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; PAS method, periodic acid Sciff method; PCD, programmed cell death; PNGase F, peptide *N*-glycosidase F; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RNS, S-like ribonucleases; ssDNA, single-stranded DNA; TILLING, targeting induced local lesions in genomes

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