Running title: Characterization of Three Rice NDPK Isozymes

Physiological and Biochemical Characterization of Three Nucleoside Diphosphate Kinase Isozymes from Rice (*Oryza sativa* L.)

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Abbreviations: NDPK, nucleoside diphosphate kinase; OsNDPK, *Oryza sativa* NDPK; AtNDPK, *Arabidopsis thaliana* NDPK; ATPD, ATPase delta gene from sweet potato; GFP, green fluorescence protein; RT-PCR, reverse transcription PCR; RFP, red fluorescence protein

Nucleoside diphosphate kinase (NDPK) is a ubiquitous enzyme that catalyzes the transfer of the γ-phosphoryl group from a nucleoside triphosphate to a nucleoside diphosphate. In this study, we examined the subcellular localization, tissue-specific gene expression, and enzymatic characteristics of three rice NDPK isozymes (OsNDPK1-OsNDPK3). Sequence comparison of the three OsNDPKs suggested differential subcellular localization. Transient expression of green fluorescence protein-fused proteins in onion cells indicated that OsNDPK2 and OsNDPK3 are localized to plastid and mitochondria respectively, while OsNDPK1 is localized to the cytosol. Expression analysis indicated that all the OsNDPKs are expressed in the leaf, leaf sheath, and immature seeds, except for OsNDPK1, in the leaf sheath. Recombinant OsNDPK2 and OsNDPK3 showed lower optimum pH and higher stability under acidic pH than OsNDPK1. In ATP formation, all the OsNDPKs displayed lower K_m values for the second substrate, ADP, than for the first substrate, NTP, and showed lowest and highest K_m values for GTP and CTP respectively.

Key words: nucleoside diphosphate kinase; subcellular localization; ping-pong mechanism; gene expression; enzyme characteristics

Nucleoside diphosphate kinase (NDPK; EC 2.7.4.6)[1, 2] is a ubiquitous enzyme that catalyzes the transfer of the γ-phosphoryl group from nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP) through a ping-pong mechanism.[3] Since it catalyzes reversible reactions, it has been considered a housekeeping enzyme that regulates nucleotide pools within the cells. However, recent studies have

suggested that NDPKs play more specific roles in signal transduction in diverse organisms, from bacteria to mammals. For instance, an NDPK is involved in the regulation of G protein activity by changing the availability of GTP in the vicinity of G protein.[4,5] Genes encoding NDPK are associated with the suppression of tumor metastasis[6] and *Drosophila* development.[7] Furthermore, a function as a transcription factor that regulates c-*myc* gene expression has been demonstrated for a human NDPK.[8]

Three types of NDPKs have been identified in plants based on their amino acid sequences.[9-14] Type I NDPK is a cytosolic protein,[11,14] while type II and type III NDPK bear an N-terminal extension likely to be involved in targeting of the proteins to chloroplasts[15-18] or mitochondria.[19,20] Plant NDPKs have also been found to be multi-functional proteins, and to be strongly related to signal transduction, differentiation, and development. *Arabidopsis* type II NDPK (AtNDPK2) regulates auxin action[21] and phytochrome-mediated signal transduction.[9] AtNDPK2 is thought to regulate the activity of G protein, since AtNDPK2 directly interacts with pea small G-proteins, Pra2 and Pra3, and controls their activities.[22]

In rice, only type I NDPK (OsNDPK1) has been characterized to date. The amount of the OsNDPK1 protein in the embryo increases during imbibition, while it decreases in the endosperm.[23] OsNDPK1 has been implicated in the cell elongation process in coleoptiles. The activity of NDPK in coleoptiles correlates with their length in *NDPK1* knock-down mutants.[24] Furthermore, *NDPK1* expression was strongly induced by a plant pathogen (*Xanthomonas oryzae* pv. *oryzae*), salicylic acid, jasmonic acid, and abscisic acid.[25]

The three-dimensional structure of OsNDPK1 has been solved in a

multimeric form (two hexamers).[26] The OsNDPK1 monomer consists of a four-stranded anti-parallel β–sheet, surrounded by six α-helices. The nucleotide-binding site in a cleft is formed by an area of highly positive potential. Two crucial, conserved active site residues, Phe58 and His115, are located on αA and β4 respectively (the names of structural elements used here follow a report of Huang *et al.*[26]).

In spite of extensive studies on the structure and physiological functions of OsNDPK1, the enzymatic properties of rice NDPK isozymes, including OsNDPK1, have not been elucidated in detail. Here, we report subcellular localization, gene expression, and enzymatic properties of three OsNDPKs.

Materials and Methods

Plant materials. Rice plants (*Oryza sativa* L. cv. Kitaake) were grown in the experimental field of Hokkaido University. Tissues from rice plants at the grain-filling period were collected and stored at -80°C until analysis.

Cloning of genes encoding OsNDPKs. Three OsNDPK genes, OsNDPK1 (Os07g0492000), OsNDPK2 (Os12g0548300), and OsNDPK3 (Os05g0595400), are predicted in the rice genome according to the rice annotation project database. The cDNAs of these genes were amplified by reverse transcription PCR (RT-PCR) from immature seeds, as follows: Total RNA was extracted from immature seeds using ISOGEN (Nippon Gene, Tokyo), and reverse transcription was performed with BcaBEST RNA PCR Kit (Takara, Shiga, Japan)

using an oligo dT primer (Takara). RT-PCR was performed with PrimeSTAR HS DNA polymerase (Takara) and the following primers: for OsNDPK1, 5'-ATCCTCCTCATCGCCTCCGCTTCTG-3' (sense) and 5'-CTTGGATGAACCTAGGGAAAGAGCATG-3' (antisense); for OsNDPK2, 5'-TCATCATCTTCTTCTTCTCCGCTCTCC-3' (sense) and 5'-CACCTCTGCTGGGAGCTATAGTACAAC-3' (antisense); and for OsNDPK3, 5'-GTACATACTAGTACAAGACTGGAGGGC-3' (sense) and 5'-TGATTACTCAGTGCACGCTCTACAATC-3' (antisense). Amplified DNA fragments were cloned into pBluescript SK II (Stratagene, La Jolla, CA) using DNA Ligation Kit Mighty Mix (Takara), and were sequenced with an ABI PISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with a Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems). Propagation of plasmid DNA was performed in *Escherichia coli* DH5α, and plasmid DNA was purified by alkaline lysis methods.[27)]

Transient expression of OsNDPKs fused with green fluorescent protein (GFP). By PCR, *Bam*HI and *Nde*I sites were introduced into the 5'- and 3'-termini of the OsNDPK genes using the following primers (*Bam*HI and *Nde*I sites underlined): for OsNDPK1, 5'-GTTCTTTGGATCCATGGAGCAGTC-3' (sense) and 5'-GCGCGAACATATGAGACTCATAGATCCAAG-3' (antisense); for OsNDPK2, 5'-GAGAAGCAGCTGGATCCATGGACGCC-3' (sense) and 5'-GCACTGATCTGGGACATATGCTCTACAAG-3' (antisense); and for OsNDPK3, 5'-CCCTAGCCAAGGATCCATGAGCAAGC-3' (sense) and 5'-GGGAGATGATTCGCCATATGGTTGACCC-3' (antisense). The GFP gene was isolated from the 35S-sGFP plasmid, the kind gift of Dr. Yasuo Niwa (University of Shizuoka), by double digestion of *Nde*I and

*Sac*I, and was fused to the OsNDPK genes on binary vector pTH-1, a derivative of pBE2113.[28] The plastidial localization marker, AtCHL27:RFP, was created by inserting *Arabidopsis* CHL27 cDNA[29] into the pH7RWG2 vector.[30] The mitochondrial marker construct, ATPD:RFP, which utilizes the signal peptide of sweet potato ATPase delta,[31] was also prepared with pH7RWG2.

Introduction of expression vectors into onion epidermal cell layer was performed by particle bombardment.[32] Gold particles coated with 2.5 μg of the respective plasmid(s) were delivered into onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad, Hercules, CA) with a rupture setting of 1,100 psi. After bombardment, the epidermal cell layer was incubated on Murashige and Skoog medium[33] at 25ºC overnight. Subcellular localization of the GFP-fusion proteins was visualized by fluorescence microscopy DM6000B (Leica, Solms, Germany). Images were processed with imaging software FW4000 (Leica).

Expression analysis of OsNDPK genes. The transcription levels of *OsNDPKs* in the leaf, leaf sheath, and immature seeds were examined by RT-PCR. Total RNA from these tissues was extracted, and first-strand cDNA was synthesized as described above. PCR was conducted with the following primers: for OsNDPK1, 5'-GCCTCCTGAGTCACCATTATAATTCTG-3' (sense) and 5'-CTTGGATGAACCTAGGGAAAGAGCATG-3' (antisense); for OsNDPK2, 5'-CCGATTACTCTCTGTTGTCAAATCTCC-3' (sense) and 5'-CACCTCTGCTGGGAGCTATAGTACAAC-3' (antisense); and for OsNDPK3, 5'-CATCTCCCCTGTTTGTTTTGTTTTTTTTTC-3' (sense) and 5'-TGATTACTCAGTGCACGCTCTACAATC-3' (antisense).

Twenty-eight, 30, and 27 cycles (98ºC for 5 s, 55ºC for 5 s, and 72ºC for 1 min) for OsNDPK1, 2, and 3 respectively were performed. Ubiquitin mRNA was used as internal control with a pair of primers: sense, 5'-CCAGGACAAGATGATCTGCC-3'; antisense, 5'-CCAGTCCATGAACCCGGCGAGTGACG-3'.

Construction of expression plasmids for OsNDPKs. Plasmid vector pET23a (Novagen, Darmstadt, Gemany) was used to produce recombinant OsNDPKs in *E. coli* BL21 (DE3) pLys. Recombinant OsNDPK2 and 3 were produced as N-terminal-truncated forms starting from the 73th and 88th amino acids respectively. Recombinant OsNDPK1 was produced in intact form. A histidine tag from the vector consisting of six histidine residues was attached to the C-terminal of each recombinant protein and utilized for affinity chromatography.

To clone the cDNA of OsNDPKs into pET23a, *Nhe*I and *Xho*I sites were introduced into the 5'- and 3'-termini of the cDNA by PCR with the following primer sets (*Nhe*I and *Xho*I sites underlined): for OsNDPK1, 5'-GAGAGATGGCTAGCTCCTTCATCATG-3' (sense) and 5'- GGCGCGAACTCGAGAGACTCATAGATCC-3' (antisense); for OsNDPK2, 5'-CGTCGTCGGTTGCTAGCTCCTATATTATG-3' (sense) and 5'-GCACTGATCTGGGACTCGAGCTCTACAAG-3' (antisense); and for OsNDPK3, 5'-CGCATGCTGCAGCTAGCGAGCGCACC-3' (sense) and 5'-GAGATGATTCGCCTCGAGGTTGACCCC-3' (antisense). The amplified DNA fragments and pET23a were digested with *Nhe*I and *Xho*I and ligated as described above.

Production and purification of recombinant OsNDPKs. Recombinant OsNDPKs were produced in *E. coli* BL21 (DE3) pLys cells carrying

the expression plasmids. The transformants were cultured at 37ºC in 1 L of Luria-Bertani medium with 100 μg/mL ampicillin until A_{600} reached 0.8. Then 1 mL of 0.1 M isopropyl β-thiogalactopyranoside was added to the culture, and the cells were grown further at 20ºC for 16 h. The bacterial cells were harvested by centrifugation and suspended in 50 mL of 20 mM Tris-HCl buffer (pH 7.4) containing 20 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. They were disrupted by sonication with Sonifier 450 (Branson, Danbury, CT), and the lysate was centrifuged to remove cell debris. The cell-free extract obtained was applied to Ni-chelating column chromatography using Chlating Sepahrose Fast Flow (GE healthcare, Uppsala, Sweden). The adsorbed protein was eluted with a linear gradient of imidazole (20-500 mM) in 20 mM Tris-HCl buffer (pH 7.4) with 10% glycerol after thorough washing with 20 mM Tris-HCl buffer (pH 7.4) containing 20 mM imidazole. The purity of the recombinant OsNDPK isozymes was confirmed by SDS-PAGE.[34] The collected fraction was dialyzed against 20 mM Tris-HCl buffer (pH 7.4) with 10% glycerol, and then more glycerol was added to 40% before storage at -20ºC. The protein concentration of purified enzymes was measured by the Bradford method,[35] and bovine serum albumin F-V (Nakalai Tesque, Kyoto, Japan) was used as standard.

Enzyme assay. NDPK activity was determined based on the initial velocity of ATP formation from ADP and GTP. A reaction mixture of 100 μL consisting of an appropriate concentration of the enzyme, 2 mM ADP (Sigma-Aldrich, St. Louis, MO), 2 mM GTP (Wako, Osaka, Japan), 4 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, and 25 mM $MgCl_2$ was incubated at 25ºC for 10 min. The enzymatic reaction was

stopped by boiling for 5 min, and the ATP produced was measured by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (φ4.6 x 50 mm, Shiseido, Tokyo); column temperature, 40ºC; injection volume, 20 μL; detection, A_{254}; flow rate, 0.8 mL/min; elution, linear gradient of 0-300 mM ammonium phosphate dibasic (pH 3.0). As standard, 0-2.0 mM ATP (Sigma-Aldrich) was used.

The optimum pH values of the OsNDPKs were determined by measuring ATP-forming velocities at the indicated pH values. Fifty mM sodium acetate buffer (pH 4.0 and 5.0), 50 mM MES-NaOH buffer (pH 5.5-7.0), 50 mM glycine-NaOH buffer (pH 10.0 and 11.0) were used as the reaction buffer.

The temperature and pH stabilities of the OsNDPKs were evaluated based on residual activity after temperature and pH treatments. The range of pH and temperature, where the enzymes retained more than 90% of original activity after treatment, were considered to be stable ranges. Temperature treatment was done by incubation of OsNDPK solution (OsNDPK1, 29 ng/mL; OsNDPK2, 17 ng/mL; and OsNDPK3, 47 ng/mL) at 10-70ºC for 15 min. For pH treatment, OsNDPKs (OsNDPK1, 0.9 mg/mL; OsNDPK2, 1.8 mg/mL; and OsNDPK3, 1.4 mg/mL) were diluted 10-fold with 0.5 M Britton-Robinson buffer (pH 2.0-12.0) and incubated at 4ºC for 16 h.

The kinetic parameters of the OsNDPKs for ADP and various NTPs (GTP, TTP, and CTP) were calculated based on the following reaction equation for the ping-pong mechanism:

$$v=k_{cat}\cdot a\cdot b/(K_{mb}\cdot a+K_{ma}\cdot b+a\cdot b)$$

where v is the reaction rate, k_{cat} is the molecular activity, a and b are concentrations of NTP and ADP respectively, and K_{ma} and K_{mb} are the affinity constants for NTP and ADP respectively. The initial velocities

of ATP formation at various concentrations of ADP (0.031-0.083 mM) and NTP (0–1 mM) were measured under the conditions described above. The reactions toward TTP and CTP were also analyzed with HPLC as the reaction to GTP.

Results

Phylogenetic analysis of the OsNDPKs

Three OsNDPK genes, OsNDPK1-OsNDPK3, are predicted in the rice genome. A comparison of the amino acid sequences of these OsNDPKs revealed that in addition to a highly conserved C-terminal region (about 50% identity), OsNDPK2 and OsNDPK3 contained putative signal sequences at the N-terminus. Phylogenetic analysis was done with the sequences of the conserved regions available for *O. sativa*, *A. thaliana*, *Zea mays*, *Pinus sativum*, *Arachis hypogaea*, *Nicotiana tabacum*, and *Brassica rapa*. The NDPKs divided into three types, and OsNDPK1-OsNDPK3 fell into types I-III respectively (Fig. 1). Fig. 1

Subcellular localization of the OsNDPKs

In order to determine the specific functions of the OsNDPKs, subcellular localization was determined using GFP-fused proteins. The full-length cDNAs of the respective NDPKs were amplified by RT-PCR, and the GFP gene was fused to the C-terminus.

The fusion genes were transiently expressed in onion epidermal cells. The fluorescence signals of the OsNDPK1:GFP protein were detected in the nucleus and the cytoplasm (Fig. 2A). In contrast, the

OsNDPK2:GFP and OsNDPK3:GFP proteins were detected as dot-like subcellular structures (Fig.2B and E). Co-expression experiments with marker genes for plastidial (AtCHL27:RFP) and mitochondrial (ATPD:RFP) targeting indicated localization of OsNDPK2:GFP and OsNDPK3:GFP to plastids and mitochondria respectively (Fig. 2D and G).

Expression of the OsNDPK genes in various tissues

Expression of the *OsNDPKs* was determined in leaf, leaf sheath, and immature seeds by RT-PCR (Fig. 3). The expression level of *OsNDPK1* was almost the same in leaf and immature seed, but was fairly low in the leaf sheath. In the case of *OsNDPK2*, high transcript levels were observed in the leaf and leaf sheath, and a lower level was detected in immature seed. *OsNDPK3* was strongly expressed in every tissue examined, and no difference in expression level was observed, unlike other isozymes.

Fig. 3

Production and purification of recombinant OsNDPKs

Recombinant OsNDPKs were produced as C-terminal His-tag proteins. For OsNDPK2 and OsNDPK3, the N-terminal hydrophobic regions (Met1-Ala72 and Met1-Ser87 respectively) were deleted because the corresponding full-length proteins were predominantly produced in the insoluble fractions (data not shown). Recombinant OsNDPKs were purified from *E. coli* cell-free extracts to homogeneity by Ni-chelating column chromatography (Fig. 4). The molecular masses of OsNDPK1- OsNDPK3, estimated by SDS-PAGE, corresponded to the theoretical masses calculated from the amino acid sequences (17.9, 17.4, and 18.0 kDa respectively).

Enzymatic properties of recombinant OsNDPKs

The recombinant OsNDPKs were characterized based on ATP-forming activity from ADP and GTP. OsNDPK1 and OsNDPK2 were stable up to 25ºC. On the other hand, OsNDPK3 was stable up to 30ºC (Fig. 5). OsNDPK1 showed the highest activity at pH 8.4, while the optimum pH values for both OsNDPK2 and OsNDPK3 were 6.2 (Fig. 6). The stable pH ranges for OsNDPK1-OsNDPK3 were different, as follows: OsNDPK1, 7.0-9.0; OsNDPK2, 6.0-9.0; and OsNDPK3, 4.0-9.0 (Fig. 7).

The kinetic parameters of the OsNDPKs for ATP formation from ADP and NTP were determined by double reciprocal plots (Table 1). Figure 8 shows the lines obtained from the initial velocities of ATP formation at various concentrations of GTP and ADP. The lines for respective ADP concentrations were parallel in all the OsNDPKs, indicating that NDPKs obeyed the ping-pong mechanism, as reported for human NDPK.[3] All the OsNDPKs displayed lower K_m values for the second substrate, ADP, than for the first substrate, NTP, and showed lowest and highest K_m values for GTP and CTP respectively (Table 1). OsNDPK1 exhibited higher K_m values than OsNDPK2 and OsNDPK 3 for all the substrates examined.

Discussion

Plant NDPKs are involved in multiple signaling steps in the regulation of differentiation and development. In rice, the physiological functions and three-dimensional structure of OsNDPK1 have been elucidated.[23-26] But the enzymatic characteristics of plant NDPKs, including OsNDPKs, are poorly understood. Here, we

investigated the enzymatic properties of three OsNDPKs along with subcellular localization and gene expression.

Phylogenetic analysis indicated that plant NDPKs are to be classified into three structural groups (types I-III) (Fig.1). Phylogenetic analysis also suggested that these three groups of NDPKs diverged before monocot and dicot separation. OsNDPK1-OsNDPK3 represent each group, suggesting that each OsNDPK has a distinctive function that is conserved among plant species.

The subcellular localization of type II and type III NDPKs in plants is controversial. AtNDPK2 was first reported as a cytosolic protein that interacts with phytochrome A[9] and several other cytosolic proteins including mitogen-activated protein kinases[36] and subgroup 3 sucrose-nonfermenting-related kinases.[37] However, a later report confirmed that AtNDPK2 is localized to the chloroplasts.[15] Furthermore, a type II NDPK from pea was purified from the leaf chloroplast.[16] Localization of AtNDPK3 is also unclear, because three groups have reported differently that AtNDPK3 is localized to the mitochondria, the chloroplasts, and both.[20,38,39] Alignment of the OsNDPK sequences indicated that OsNDPK2 and OsNDPK3 contain a putative N-terminal targeting sequence, while the C-terminal of the three OsNDPKs are well conserved (data not shown). The N-terminal targeting sequences appear to have features typical of both plastidial and mitochondrial localization. Hence, prediction servers returned different localization results. For example, TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicted plastid localization for both OsNDPK2 and OsNDPK3, while PSORT (http://psort.hgc.jp/) predicted mitochondrial localization. According to our data for the C-terminal GFP fusion proteins, the subcellular

localizations of OsNDPK2 and OsNDPK3 were clearly distinguished. OsNDPK2 was localized to the plastids (Fig.2B) and OsNDPK3 to the mitochondria (Fig. 2E).

The recombinant OsNDPK2 and OsNDPK3 showed higher activity and stability at lower pH values than OsNDPK1 (Figs. 6 and 7). This can be attributed to adaptation to the localized organelle, plastids, and mitochondria respectively, in which the pH values are more labile than that of cytosol due to excess accumulation of protons. Kinetic analysis of the OsNDPKs revealed that all the isozymes exhibited lowest affinity constants for GTP among NTP (Table 1). The higher preference for GTP suggests a possible role of OsNDPKs in maintaining GTP levels. Such a mechanism might be involved in regulatory processes, as found for *Drosophila*, in which NDPK controls GTP levels in the vicinity of a G protein that regulates cell signaling.[4, 5)]

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Figure legends

Fig. 1. Phylogenetic Tree of Plant NDPKs.

A phylogenetic tree of plant NDPKs was constructed using the Clustal X program (ver. 2.0.11), and visualized with TreeView 1.6.6 software. Numbers in the figure indicate bootstrap values. Os, *Oryza sativa*; At, *Arabidopsis thaliana*; Zm, *Zea mays*; Ps, *Pinus sativum*; Ah, *Arachis hypogaea*; Nt, *Nicotiana tabacum*; Br, *Brassica rapa*.

Fig. 2. Subcellular Localization of OsNDPKs in Onion Epidermal Cells.

A, Fluorescent images of an onion cell expressing OsNDPK1:GFP. B-D, fluorescent images of an onion cell co-expressing OsNDPK2:GFP and AtCHL27:RFP. E-G, fluorescence images of an onion cell co-expressing OsNDPK3:GFP and ATPD:RFP. B, GFP fluorescence image of OsNDPK2; C, RFP fluorescence image of AtCHI27; D, overlay image of B and C; E, GFP fluorescence image of OsNDPK3; F, RFP fluorescence image of ATPD; G, overlay image of E and F. Scale bars are 20 μm.

Fig. 3. RT-PCR Analysis for Gene Expression of *OsNDPKs* in Leaf, Leaf Sheath, and Immature Seed Tissues.

Semi-quantitative RT-PCR was performed to evaluate the expression levels of *OsNDPKs* in leaf, leaf sheath, and immature seed tissues. The ubiquitin gene was used as control.

Fig. 4. SDS-PAGE Analysis of Purified Recombinant OsNDPKs.

Purified enzyme (2 μg) was separated on a 15% w/v polyacrylamide gel. The molecular masses of standard proteins are shown on the left. M, size marker; 1, OsNDPK1; 2, OsNDPK2; 3, OsNDPK3.

Fig. 5. Temperature Stability of Recombinant OsNDPKs.

The residual activities of recombinant OsNDPK1-OsNDPK3 were measured after 15 min incubation at indicated temperatures. Solid circle, hollow circle, and solid triangle indicate OsNDPK1-OsNDPK3 respectively. Data are the mean ± SD for three independent experiments.

Fig. 6. pH Activity Curves of Recombinant OsNDPKs.

The reaction velocities of recombinant OsNDPK1-OsNDPK3 at indicated pH values were measured. Solid circle, hollow circle, and solid triangle indicate OsNDPK1-OsNDPK3 respectively. Data are the mean ± SD for three independent experiments.

Fig. 7. pH Stability of Recombinant OsNDPKs.

The residual activities of recombinant OsNDPK1-OsNDPK3 were measured after pH treatment at 4ºC for 16 h. Solid circle, hollow circle, and solid triangle indicate OsNDPK1-OsNDPK3 respectively. Data are the mean ± SD for three independent experiments.

Fig. 8. Lineweaver-Burk Plots for the Reaction of Recombinant OsNDPKs toward ADP and GTP.

The double reciprocal plots were obtained from the initial velocities of recombinant OsNDPKs to various concentrations of ADP and GTP. Hollow triangle, solid triangle, hollow circle, and solid circle indicate

the 1/*v* values for 0.042, 0.05, 0.063, and 0.083 mM ADP respectively. GTP concentrations were 0.25, 0.33, 0.5, and 1.0 mM. A, OsNDPK1; B, OsNDPK2; C, OsNDPK3. Data are the mean ± SD for three independent experiments.

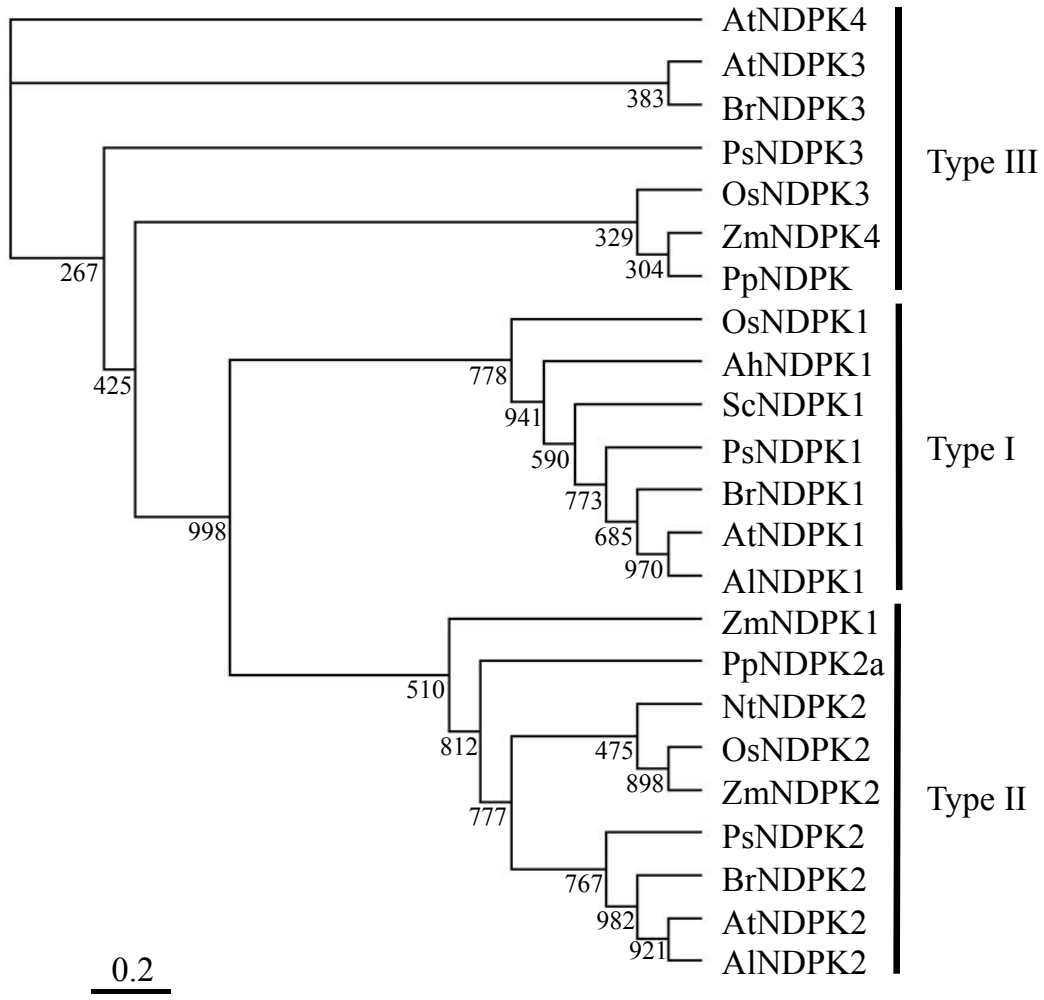

Fig. 1, Kihara *et al*.

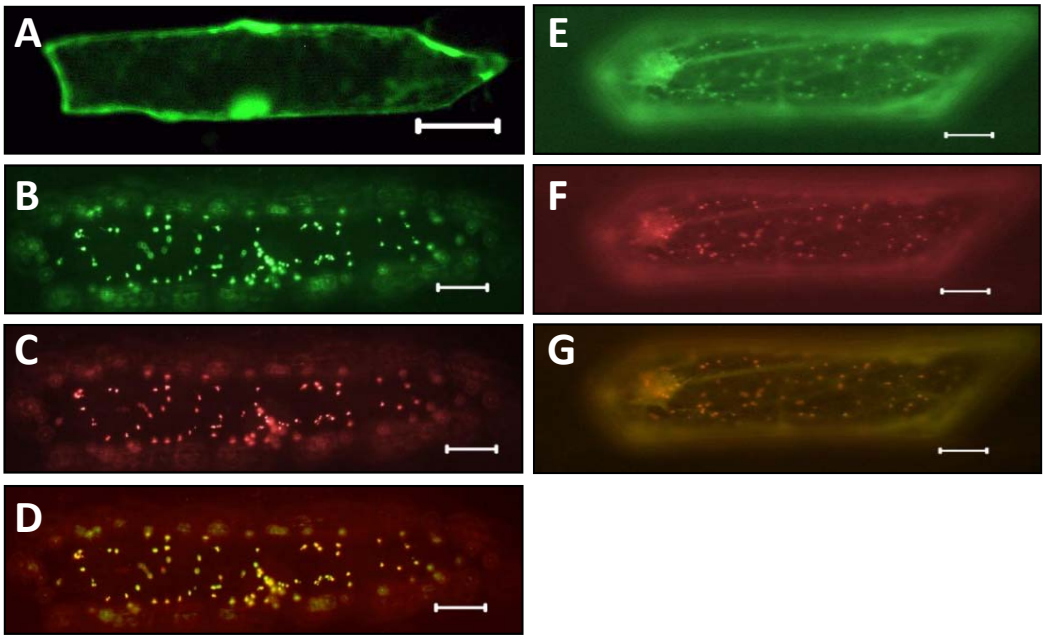

Fig. 2, Kihara *et al*.
Color print

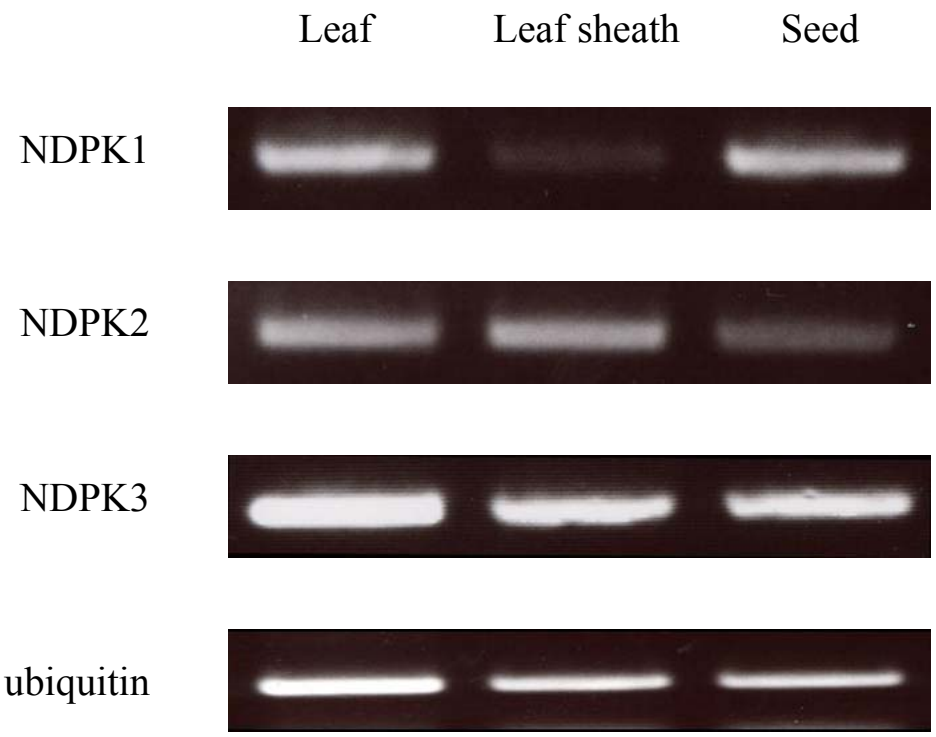

Fig. 3, Kihara *et al*.

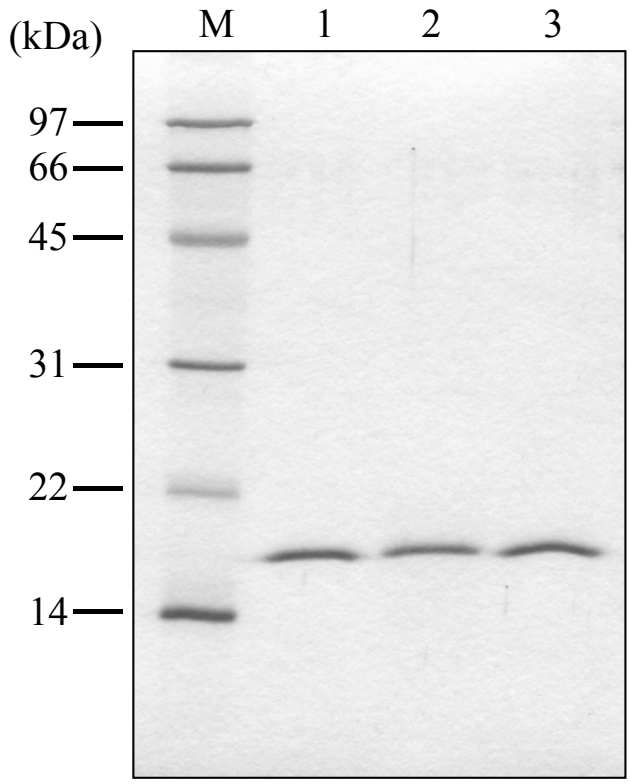

Fig. 4, Kihara *et al*.

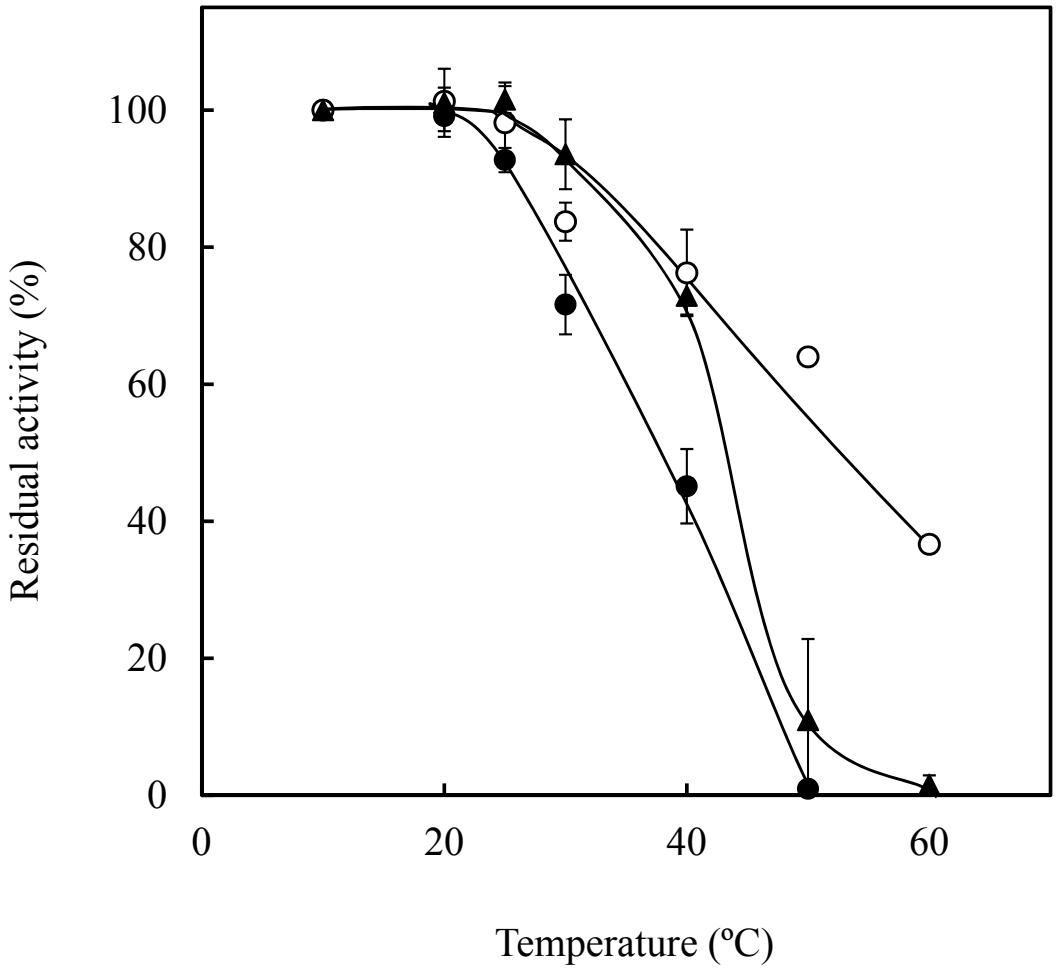

Fig. 5, Kihara *et al*.

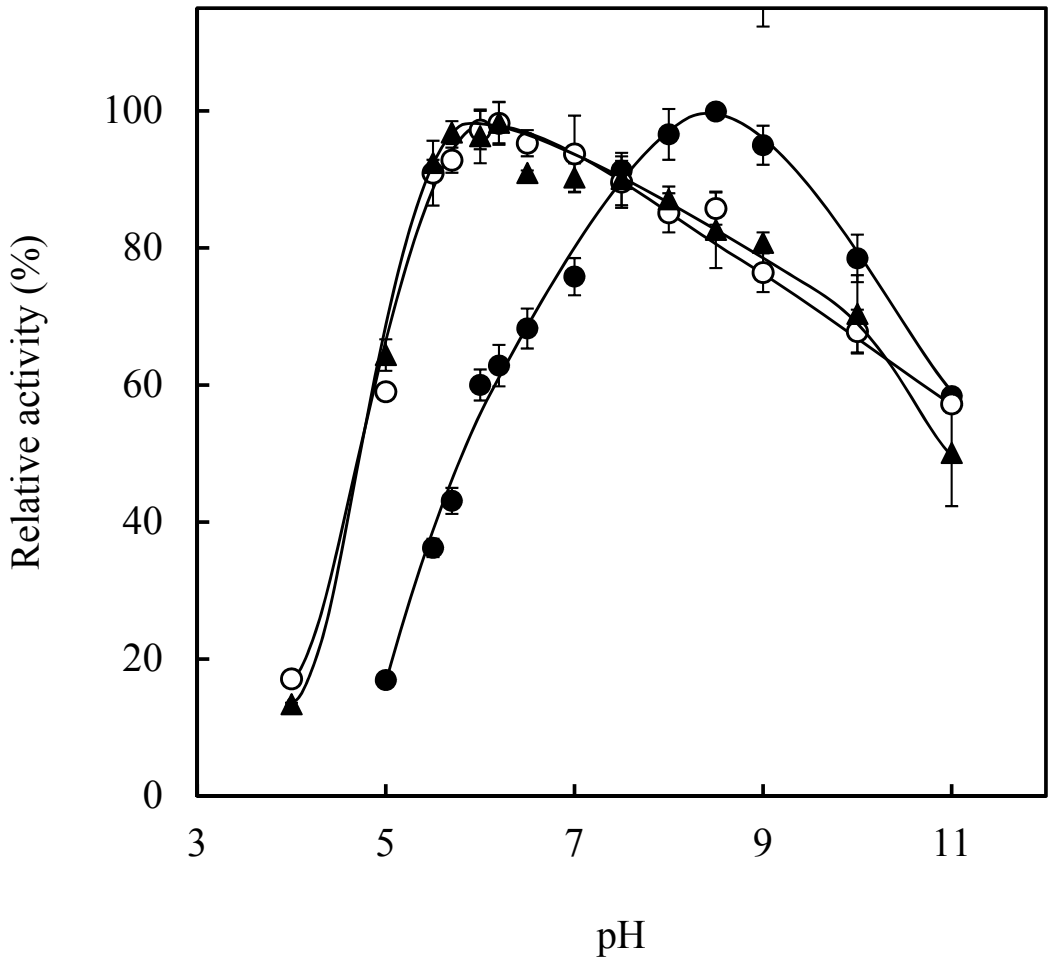

Fig. 6, Kihara *et al*.

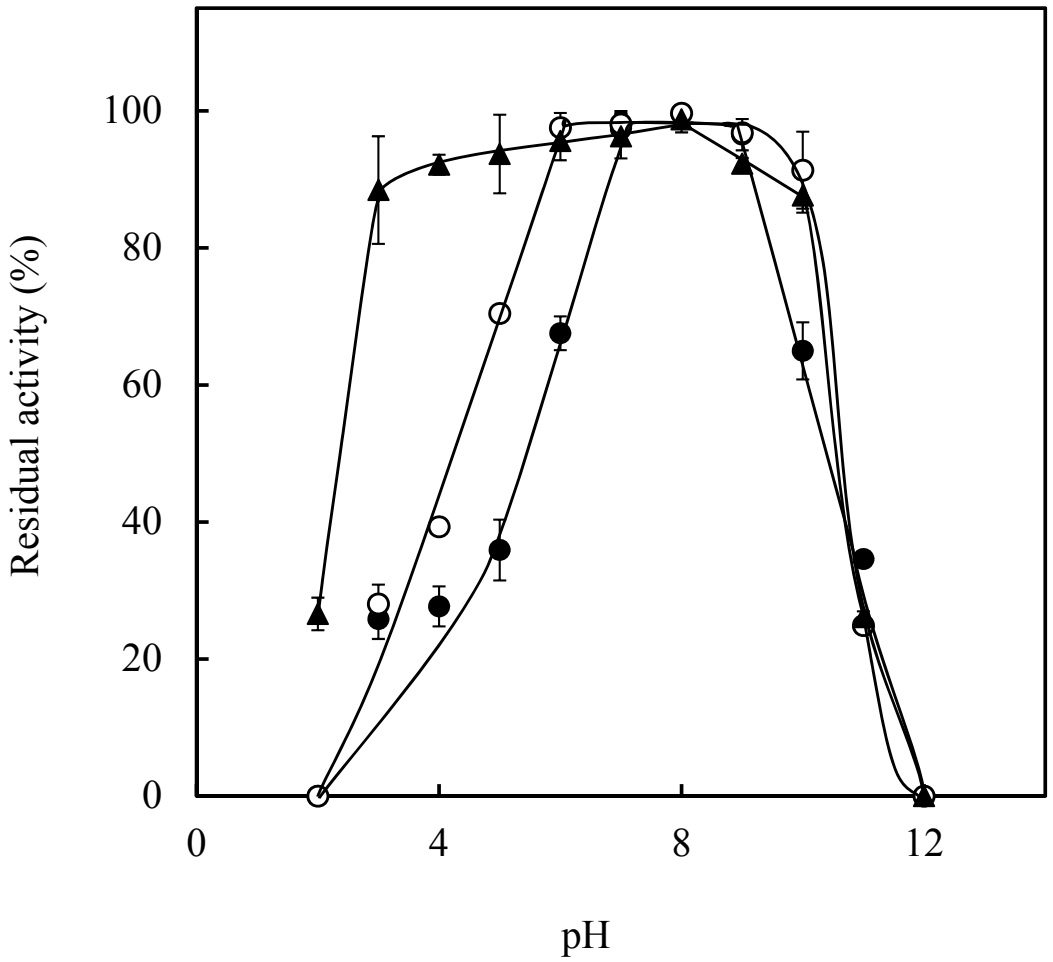

Fig. 7, Kihara *et al*.

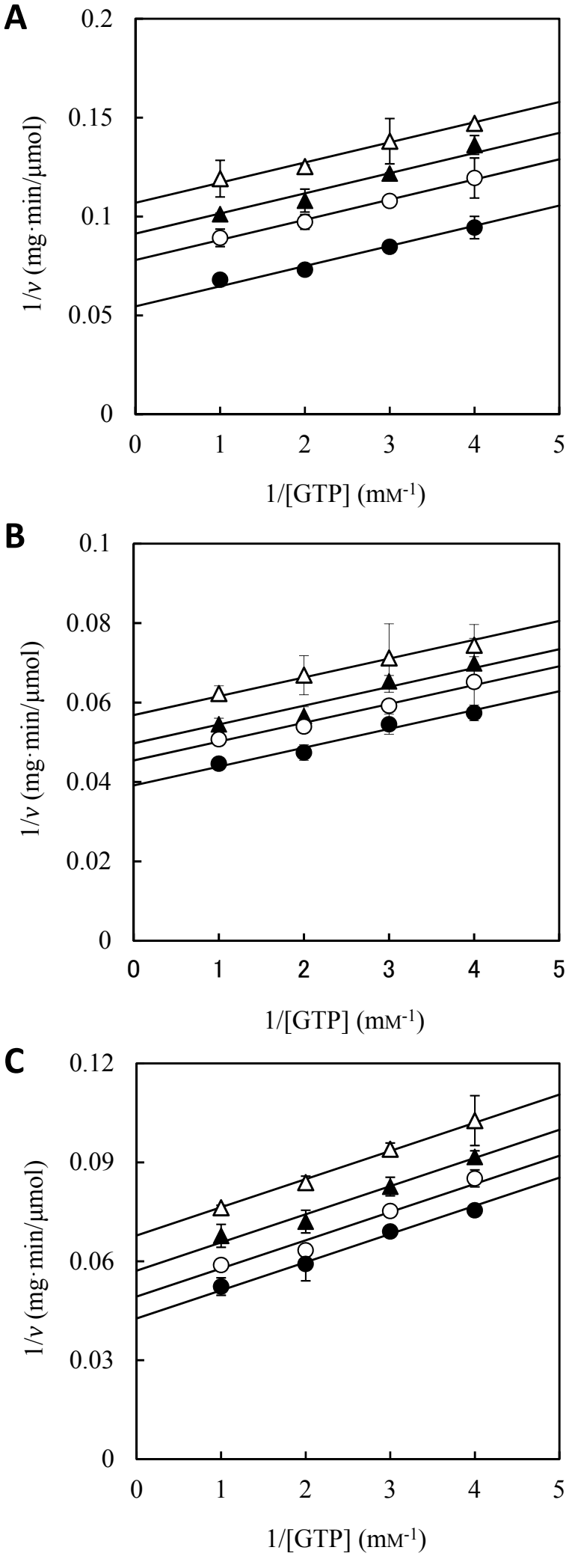

Fig. 8, Kihara *et al*.

Table 1. Kinetic Parameters of Recombinant OsNDPKs

(a) OsNDPK1

Substrate	k_{cat} (s^{-1})	K_{ma}[a] (mM)	K_{mb}[b] (mM)	k_{cat}/K_{ma} ($s^{-1}mM^{-1}$)	k_{cat}/K_{mb} ($s^{-1}mM^{-1}$)
CTPP	229	6.72	0.552	34.1	415
GTP	49.4	1.69	0.707	29.2	69.9
TTP	55.0	5.28	0.392	10.4	140

(b) OsNDPK2

Substrate	k_{cat} (s^{-1})	K_{ma}[a] (mM)	K_{mb}[b] (mM)	k_{cat}/K_{ma} ($s^{-1}mM^{-1}$)	k_{cat}/K_{mb} ($s^{-1}mM^{-1}$)
CTPP	85.2	9.37	1.26	9.10	67.6
GTP	27.7	0.450	0.138	61.6	210
TTP	14.2	0.890	0.0790	16.0	180

(c) OsNDPK3

Substrate	k_{cat} (s^{-1})	K_{ma}[a] (mM)	K_{mb}[b] (mM)	k_{cat}/K_{ma} ($s^{-1}mM^{-1}$)	k_{cat}/K_{mb} ($s^{-1}mM^{-1}$)
CTPP	89.0	5.28	0.578	16.9	154
GTP	17.9	0.510	0.125	35.1	143
TTP	33.5	1.34	0.108	25.0	310

a, Affinity constant for NTP

b, Affinity constant for ADP