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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

1 suggested that NDPKs play more specific roles in signal transduction
2 in diverse organisms, from bacteria to mammals. For instance, an
3 NDPK is involved in the regulation of G protein activity by changing
4 the availability of GTP in the vicinity of G protein.^{4,5)} Genes encoding
5 NDPK are associated with the suppression of tumor metastasis⁶⁾ and
6 *Drosophila* development.⁷⁾ Furthermore, a function as a transcription
7 factor that regulates *c-myc* gene expression has been demonstrated for
8 a human NDPK.⁸⁾

9 Three types of NDPKs have been identified in plants based on their
10 amino acid sequences.⁹⁻¹⁴⁾ Type I NDPK is a cytosolic protein,^{11,14)}
11 while type II and type III NDPK bear an N-terminal extension likely to
12 be involved in targeting of the proteins to chloroplasts¹⁵⁻¹⁸⁾ or
13 mitochondria.^{19,20)} Plant NDPKs have also been found to be multi-
14 functional proteins, and to be strongly related to signal transduction,
15 differentiation, and development. *Arabidopsis* type II NDPK
16 (AtNDPK2) regulates auxin action²¹⁾ and phytochrome-mediated signal
17 transduction.⁹⁾ AtNDPK2 is thought to regulate the activity of G
18 protein, since AtNDPK2 directly interacts with pea small G-proteins,
19 Pra2 and Pra3, and controls their activities.²²⁾

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

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

1 multimeric form (two hexamers).²⁶⁾ The OsNDPK1 monomer consists
2 of a four-stranded anti-parallel β -sheet, surrounded by six α -helices.
3 The nucleotide-binding site in a cleft is formed by an area of highly
4 positive potential. Two crucial, conserved active site residues, Phe58
5 and His115, are located on αA and $\beta 4$ respectively (the names of
6 structural elements used here follow a report of Huang *et al.*²⁶⁾).

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

12 13 14 **Materials and Methods**

15
16 *Plant materials.* Rice plants (*Oryza sativa* L. cv. Kitaake) were
17 grown in the experimental field of Hokkaido University. Tissues from
18 rice plants at the grain-filling period were collected and stored at -
19 80°C until analysis.

20
21 *Cloning of genes encoding OsNDPKs.* Three OsNDPK genes,
22 OsNDPK1 (Os07g0492000), OsNDPK2 (Os12g0548300), and
23 OsNDPK3 (Os05g0595400), are predicted in the rice genome
24 according to the rice annotation project database. The cDNAs of these
25 genes were amplified by reverse transcription PCR (RT-PCR) from
26 immature seeds, as follows: Total RNA was extracted from immature
27 seeds using ISOGEN (Nippon Gene, Tokyo), and reverse transcription
28 was performed with BcaBEST RNA PCR Kit (Takara, Shiga, Japan)

1 using an oligo dT primer (Takara). RT-PCR was performed with
2 PrimeSTAR HS DNA polymerase (Takara) and the following primers:
3 for OsNDPK1, 5'-ATCCTCCTCATCGCCTCCGCTTCTG-3' (sense) and
4 5'-CTTGGATGAACCTAGGGAAAGAGCATG-3' (antisense); for
5 OsNDPK2, 5'-TCATCATCTTCTTCTTCTCCGCTCTCC-3' (sense) and
6 5'-CACCTCTGCTGGGAGCTATAGTACAAC-3' (antisense); and for
7 OsNDPK3, 5'-GTACATACTAGTACAAGACTGGAGGGC-3' (sense)
8 and 5'-TGATTACTCAGTGCACGCTCTACAATC-3' (antisense).
9 Amplified DNA fragments were cloned into pBluescript SK II
10 (Stratagene, La Jolla, CA) using DNA Ligation Kit Mighty Mix
11 (Takara), and were sequenced with an ABI PISM 310 Genetic Analyzer
12 (Applied Biosystems, Foster City, CA) with a Big Dye Terminator 3.1
13 Sequencing Kit (Applied Biosystems). Propagation of plasmid DNA
14 was performed in *Escherichia coli* DH5 α , and plasmid DNA was
15 purified by alkaline lysis methods.²⁷⁾

16
17 *Transient expression of OsNDPKs fused with green fluorescent*
18 *protein (GFP).* By PCR, *Bam*HI and *Nde*I sites were introduced into
19 the 5'- and 3'-termini of the OsNDPK genes using the following
20 primers (*Bam*HI and *Nde*I sites underlined): for OsNDPK1, 5'-
21 GTTCTTTGGATCCATGGAGCAGTC-3' (sense) and 5'-
22 GCGCGAACATATGAGACTCATAGATCCAAG-3' (antisense); for
23 OsNDPK2, 5'-GAGAAGCAGCTGGATCCATGGACGCC-3' (sense) and
24 5'-GCACTGATCTGGGACCATATGCTCTACAAG-3' (antisense); and for
25 OsNDPK3, 5'-CCCTAGCCAAGGGATCCATGAGCAAGC-3' (sense) and
26 5'-GGGAGATGATTCGCCCATATGGTTGACCC-3' (antisense). The GFP
27 gene was isolated from the 35S-sGFP plasmid, the kind gift of Dr.
28 Yasuo Niwa (University of Shizuoka), by double digestion of *Nde*I and

1 *SacI*, and was fused to the *OsNDPK* genes on binary vector pTH-1, a
2 derivative of pBE2113.²⁸⁾ The plastidial localization marker,
3 *AtCHL27:RFP*, was created by inserting *Arabidopsis* CHL27 cDNA²⁹⁾
4 into the pH7RWG2 vector.³⁰⁾ The mitochondrial marker construct,
5 *ATPD:RFP*, which utilizes the signal peptide of sweet potato ATPase
6 delta,³¹⁾ was also prepared with pH7RWG2.

7 Introduction of expression vectors into onion epidermal cell layer
8 was performed by particle bombardment.³²⁾ Gold particles coated with
9 2.5 µg of the respective plasmid(s) were delivered into onion
10 epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad,
11 Hercules, CA) with a rupture setting of 1,100 psi. After bombardment,
12 the epidermal cell layer was incubated on Murashige and Skoog
13 medium³³⁾ at 25°C overnight. Subcellular localization of the GFP-
14 fusion proteins was visualized by fluorescence microscopy DM6000B
15 (Leica, Solms, Germany). Images were processed with imaging
16 software FW4000 (Leica).

17

18 *Expression analysis of OsNDPK genes.* The transcription levels of
19 *OsNDPKs* in the leaf, leaf sheath, and immature seeds were examined
20 by RT-PCR. Total RNA from these tissues was extracted, and first-
21 strand cDNA was synthesized as described above. PCR was conducted
22 with the following primers: for *OsNDPK1*, 5'-
23 GCCTCCTGAGTCACCATTATAATTCTG-3' (sense) and 5'-
24 CTTGGATGAACCTAGGGAAAGAGCATG-3' (antisense); for
25 *OsNDPK2*, 5'-CCGATTACTCTCTGTTGTCAAATCTCC-3' (sense) and
26 5'-CACCTCTGCTGGGAGCTATAGTACAAC-3' (antisense); and for
27 *OsNDPK3*, 5'-CATCTCCCCTGTTTGTGTTTTGTTTTTTTTTC-3' (sense)
28 and 5'-TGATTACTCAGTGCACGCTCTACAATC-3' (antisense).

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

6

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

15 To clone the cDNA of OsNDPKs into pET23a, *NheI* and *XhoI* sites
16 were introduced into the 5'- and 3'-termini of the cDNA by PCR with
17 the following primer sets (*NheI* and *XhoI* sites underlined): for
18 OsNDPK1, 5'-GAGAGATGGCTAGCTCCTTCATCATG-3' (sense) and
19 5'-GGCGCGAACTCGAGAGACTCATAGATCC-3' (antisense); for
20 OsNDPK2, 5'-CGTCGTCGGTTGCTAGCTCCTATATTATG-3' (sense)
21 and 5'-GCACTGATCTGGGACTCGAGCTCTACAAG-3' (antisense);
22 and for OsNDPK3, 5'-CGCATGCTGCAGCTAGCGAGCGCACC-3'
23 (sense) and 5'-GAGATGATTGCCTCGAGGTTGACCCC-3'
24 (antisense). The amplified DNA fragments and pET23a were digested
25 with *NheI* and *XhoI* and ligated as described above.

26

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

1 the expression plasmids. The transformants were cultured at 37°C in 1
2 L of Luria-Bertani medium with 100 µg/mL ampicillin until A_{600}
3 reached 0.8. Then 1 mL of 0.1 M isopropyl β -thiogalactopyranoside
4 was added to the culture, and the cells were grown further at 20°C for
5 16 h. The bacterial cells were harvested by centrifugation and
6 suspended in 50 mL of 20 mM Tris-HCl buffer (pH 7.4) containing 20
7 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. They were
8 disrupted by sonication with Sonifier 450 (Branson, Danbury, CT), and
9 the lysate was centrifuged to remove cell debris. The cell-free extract
10 obtained was applied to Ni-chelating column chromatography using
11 Chlating Sepahrose Fast Flow (GE healthcare, Uppsala, Sweden). The
12 adsorbed protein was eluted with a linear gradient of imidazole (20-
13 500 mM) in 20 mM Tris-HCl buffer (pH 7.4) with 10% glycerol after
14 thorough washing with 20 mM Tris-HCl buffer (pH 7.4) containing 20
15 mM imidazole. The purity of the recombinant OsNDPK isozymes was
16 confirmed by SDS-PAGE.³⁴⁾ The collected fraction was dialyzed
17 against 20 mM Tris-HCl buffer (pH 7.4) with 10% glycerol, and then
18 more glycerol was added to 40% before storage at -20°C. The protein
19 concentration of purified enzymes was measured by the Bradford
20 method,³⁵⁾ and bovine serum albumin F-V (Nakalai Tesque, Kyoto,
21 Japan) was used as standard.

22
23 *Enzyme assay.* NDPK activity was determined based on the initial
24 velocity of ATP formation from ADP and GTP. A reaction mixture of
25 100 µL consisting of an appropriate concentration of the enzyme, 2 mM
26 ADP (Sigma-Aldrich, St. Louis, MO), 2 mM GTP (Wako, Osaka,
27 Japan), 4 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, and 25 mM
28 MgCl₂ was incubated at 25°C for 10 min. The enzymatic reaction was

1 stopped by boiling for 5 min, and the ATP produced was measured by
2 HPLC under the following conditions: column, Capcell Pak NH₂ UG80
3 (φ4.6 x 50 mm, Shiseido, Tokyo); column temperature, 40°C; injection
4 volume, 20 μL; detection, A₂₅₄; flow rate, 0.8 mL/min; elution, linear
5 gradient of 0-300 mM ammonium phosphate dibasic (pH 3.0). As
6 standard, 0-2.0 mM ATP (Sigma-Aldrich) was used.

7 The optimum pH values of the OsNDPKs were determined by
8 measuring ATP-forming velocities at the indicated pH values. Fifty mM
9 sodium acetate buffer (pH 4.0 and 5.0), 50 mM MES-NaOH buffer (pH
10 5.5-7.0), 50 mM glycine-NaOH buffer (pH 10.0 and 11.0) were used as
11 the reaction buffer.

12 The temperature and pH stabilities of the OsNDPKs were evaluated
13 based on residual activity after temperature and pH treatments. The
14 range of pH and temperature, where the enzymes retained more than
15 90% of original activity after treatment, were considered to be stable
16 ranges. Temperature treatment was done by incubation of OsNDPK
17 solution (OsNDPK1, 29 ng/mL; OsNDPK2, 17 ng/mL; and OsNDPK3,
18 47 ng/mL) at 10-70°C for 15 min. For pH treatment, OsNDPKs
19 (OsNDPK1, 0.9 mg/mL; OsNDPK2, 1.8 mg/mL; and OsNDPK3, 1.4
20 mg/mL) were diluted 10-fold with 0.5 M Britton-Robinson buffer (pH
21 2.0-12.0) and incubated at 4°C for 16 h.

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

25
$$v = k_{cat} \cdot a \cdot b / (K_{mb} \cdot a + K_{ma} \cdot b + a \cdot b)$$

26 where v is the reaction rate, k_{cat} is the molecular activity, a and b are
27 concentrations of NTP and ADP respectively, and K_{ma} and K_{mb} are the
28 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

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

7

8 *Expression of the OsNDPK genes in various tissues*

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

Fig. 3

17

18 *Production and purification of recombinant OsNDPKs*

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

Fig. 4

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 25°C. On the other hand, OsNDPK3 was stable up to 30°C (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).

Fig. 5

Fig. 6

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.

Table 1

Fig. 8

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

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

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

9 The subcellular localization of type II and type III NDPKs in plants
10 is controversial. AtNDPK2 was first reported as a cytosolic protein
11 that interacts with phytochrome A⁹⁾ and several other cytosolic
12 proteins including mitogen-activated protein kinases³⁶⁾ and subgroup 3
13 sucrose-nonfermenting-related kinases.³⁷⁾ However, a later report
14 confirmed that AtNDPK2 is localized to the chloroplasts.¹⁵⁾
15 Furthermore, a type II NDPK from pea was purified from the leaf
16 chloroplast.¹⁶⁾ Localization of AtNDPK3 is also unclear, because three
17 groups have reported differently that AtNDPK3 is localized to the
18 mitochondria, the chloroplasts, and both.^{20,38,39)} Alignment of the
19 OsNDPK sequences indicated that OsNDPK2 and OsNDPK3 contain a
20 putative N-terminal targeting sequence, while the C-terminal of the
21 three OsNDPKs are well conserved (data not shown). The N-terminal
22 targeting sequences appear to have features typical of both plastidial
23 and mitochondrial localization. Hence, prediction servers returned
24 different localization results. For example, TargetP
25 (<http://www.cbs.dtu.dk/services/TargetP/>) predicted plastid
26 localization for both OsNDPK2 and OsNDPK3, while PSORT
27 (<http://psort.hgc.jp/>) predicted mitochondrial localization. According
28 to our data for the C-terminal GFP fusion proteins, the subcellular

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

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

18 **Acknowledgment**

19
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25 **References**

- 26
27 1) Berg P and Joklik WK, *Nature*, **172**, 1008-1009 (1953).
28 2) Krebs HA and Hems R, *Biochim. Biophys. Acta*, **1953**, 172-180

- 1 (1953).
- 2 3) Mourad N and Parks Jr RE, *J. Biol. Chem.*, **241**, 271-278 (1966).
- 3 4) Randazzo PA, Northup JK, and Kahn RA, *Science*, **254**, 850-853
- 4 (1991).
- 5 5) Randazzo PA, Northup JK, and Kahn RA, *J. Biol. Chem.*, **267**,
- 6 18182-18189 (1992).
- 7 6) Steeg PS, Bevilaqua G, Kooper L, Thorgeirsson UP, Talmadge JE,
- 8 Liotta LA, and Sobel ME, *J. Natl. Cancer Inst.*, **80**, 200-204
- 9 (1988).
- 10 7) Biggs J, Hersperger E, Steeg PS, Liotta LA, and Shean A, *Cell*,
- 11 **63**, 933-940 (1990).
- 12 8) Postel EH, Berberich SJ, Fling SJ, and Ferrone CA, *Science*, **261**,
- 13 478-480 (1993).
- 14 9) Choi G, Yi H, Lee J, Kwon Y, Sho MS, Shin B, Luka Z, Hahn T,
- 15 and Son P, *Nature*, **401**, 610-613 (1999).
- 16 10) Finan PM, White IR, Redpath SH, Findlay JBC, and Millner PA,
- 17 *Plant Mol. Biol.*, **25**, 59-67 (1994).
- 18 11) Nomura T, Yatsunami K, Honda A, Sugimoto Y, Fukui T, Zhang J,
- 19 Yamamoto J, and Ichikawa A, *Arch. Biochem. Biophys.*, **297**, 42-
- 20 45 (1992).
- 21 12) Harris N, Taylor JE, and Roberts JA, *Plant Mol. Biol.*, **25**, 739-
- 22 742 (1994).
- 23 13) Sommer P and Song PS, *Biochim. Biophys. Acta*, **1222**, 464-470
- 24 (1994).
- 25 14) Yano A, Shimazaki T, Kato A, Umeda M, and Uchimiya H, *Plant*
- 26 *Mol. Biol.*, **23**, 1087-1090 (1993).
- 27 15) Bölter B, Sharma R, and Soll J, *Planta*, **226**, 1059-1065 (2007).
- 28 16) Lubeck J and Soll J, *Planta*, **196**, 668-673 (1995).

- 1 17) Yang LM and Lamppa GK, *Biochim. Biophys. Acta*, **1294**, 99-102
2 (1996).
- 3 18) Peltier JB, Cai Y, Sun Q, Zabrouskov V, Giacomelli L, Rudella A,
4 Ytterberg AJ, Rutschow H, and van Wijk KJ, *Mol. Cell.*
5 *Proteomics*, **5**, 114-133 (2006).
- 6 19) Escobar Galvis ML, Håkansson G, Alexciev K, and Knorpp C,
7 *Biochimie*, **81**, 1089-1096 (1999).
- 8 20) Sweetlove LJ, Mowday B, Hebestreit HF, Leaver CJ, and Millar
9 AH, *FEBS Lett.*, **508**, 272-276 (2001).
- 10 21) Choi G, Kim J, Hong S, Shin B, Choi G, Blakeslee JJ, Murphy
11 AS, Seo YW, Kim K, Koh E, Song P, and Lee H, *Plant Cell*
12 *Physiol.*, **46**, 1246-1254 (2005).
- 13 22) Shen Y, Han Y, Kim J, and Song P, *BMB Rep.*, **41**, 645-650 (2008).
- 14 23) Yano A, Umeda M, and Uchimiya H, *Plant Mol. Biol.*, **27**, 1053-
15 1058 (1995).
- 16 24) Pan L, Kawai M, Yano A, and Uchiyama H, *Plant Physiol.*, **122**,
17 447-452 (2000).
- 18 25) Cho SM, Shin SH, Kim KS, Kim YC, Eun MY, and Cho BH, *Mol.*
19 *Cells*, **18**, 390-395 (2004).
- 20 26) Huang J, Chang T, Chang C, and Chen C, *J. Struct. Biol.*, **150**,
21 309-318 (2005).
- 22 27) Sambrook J and Russell DW, "Molecular Cloning, a Laboratory
23 Manual" 3rd ed., Cold Spring Harbor Laboratory Press, Cold
24 Spring Harbor (2001).
- 25 28) Mitsuhashi I, Ugaki M, Hirochika H, Ohshima M, Murakami T,
26 Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno
27 K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, and Ohashi Y,
28 *Plant Cell Physiol.*, **37**, 49-59 (1996).

- 1 29) Bang WY, Jeong IS, Kim DW, Im CH, Ji C, Hwang SM, Kim SW,
2 Son YS, Jeong J, Shiina T, and Bahk JD, *Plant Cell Physiol.*, **49**,
3 1350-1363 (2008).
- 4 30) Karimi M, Inze D, and Depicker A, *Trends Plant Sci.*, **7**, 193-195
5 (2002).
- 6 31) Kimura T, Takeda S, Asahi T, and Nakamura K, *J. Biol. Chem.*,
7 **265**, 6079-6085 (1990).
- 8 32) Klaus SMJ, Huang FC, Golds TJ, and Koop HU, *Nat. Biotechnol.*,
9 **22**, 225–229 (2004).
- 10 33) Murashige T and Skoog F, *Physiol. Plant*, **15**, 473-497 (1962).
- 11 34) Laemmli UK, *Nature*, **227**, 680-685 (1970).
- 12 35) Bradford MM, *Anal. Biochem.*, **72**, 248-254 (1976).
- 13 36) Moon H, Lee B, Choi G, Shin D, Prasad DT, Lee O, Kwak S, Kim
14 DH, Nam J, Bahk J, Hong JC, Lee SY, Cho MJ, and Lim CO,
15 *Proc. Natl. Acad. Sci. USA*, **100**, 358-363 (2003).
- 16 37) Verslues PE, Batelli G, Grillo S, Agius F, Kim Y, Zhu J, Agarwal
17 M, Katiyar-Agarwal S, and Zhu J, *Mol. Cell. Biol.*, **27**, 7771-7780
18 (2007).
- 19 38) Spetea C, Hundal T, Lundin B, Heddad M, Adamska I, and
20 Andersson B, *Proc. Natl. Acad. Sci. USA*, **101**, 1409-1414 (2004).
- 21 39) Hammargren J, Rosenquist S, Jansson C, and Knorpp C, *Plant*
22 *Cell Rep.*, **27**, 529-534 (2008).

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.

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

4
5 **Fig. 5.** Temperature Stability of Recombinant OsNDPKs.

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

11

12 **Fig. 6.** pH Activity Curves of Recombinant OsNDPKs.

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

17

18 **Fig. 7.** pH Stability of Recombinant OsNDPKs.

19 The residual activities of recombinant OsNDPK1-OsNDPK3 were
20 measured after pH treatment at 4°C for 16 h. Solid circle, hollow
21 circle, and solid triangle indicate OsNDPK1-OsNDPK3 respectively.
22 Data are the mean $\pm$ SD for three independent experiments.

23

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

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

1 the $1/v$ values for 0.042, 0.05, 0.063, and 0.083 mM ADP respectively.
2 GTP concentrations were 0.25, 0.33, 0.5, and 1.0 mM. A, OsNDPK1; B,
3 OsNDPK2; C, OsNDPK3. Data are the mean $\pm$ SD for three
4 independent experiments.
5

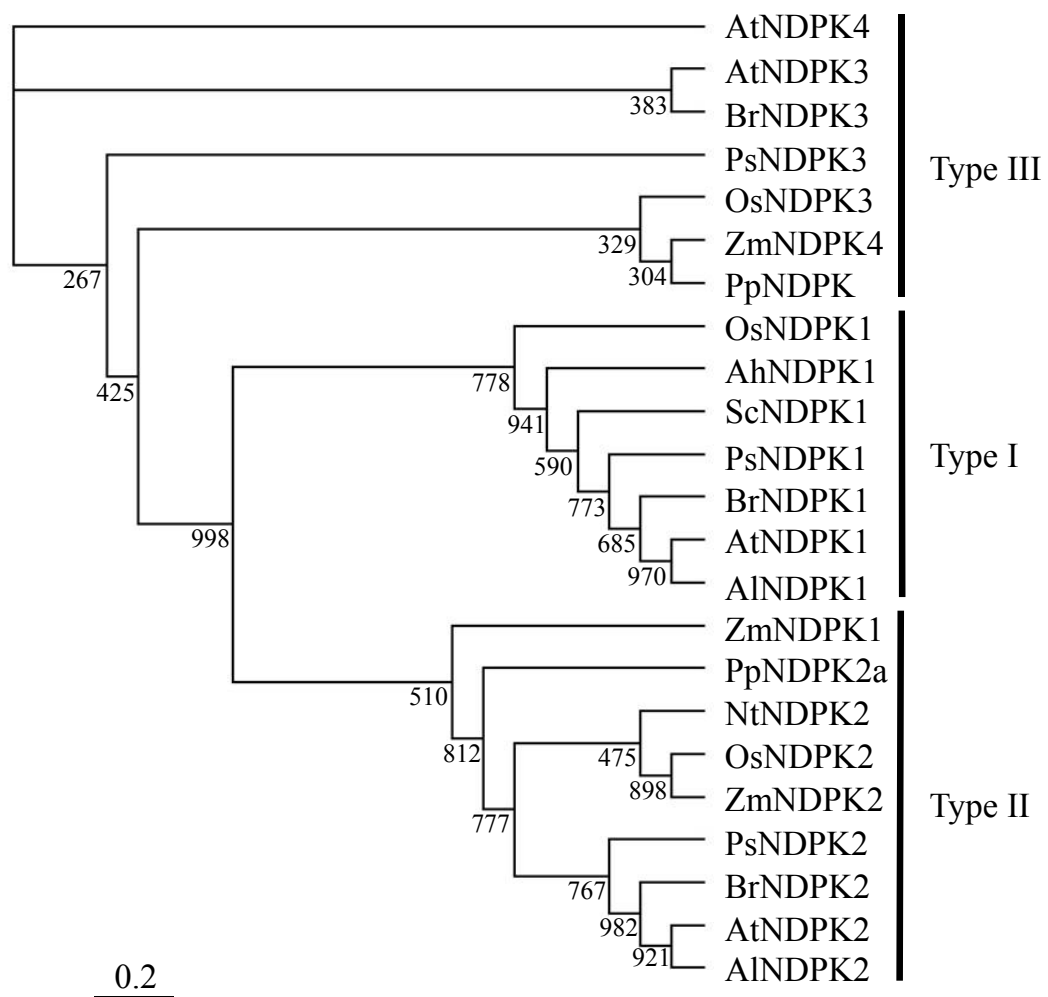


Fig. 1, Kihara *et al.*

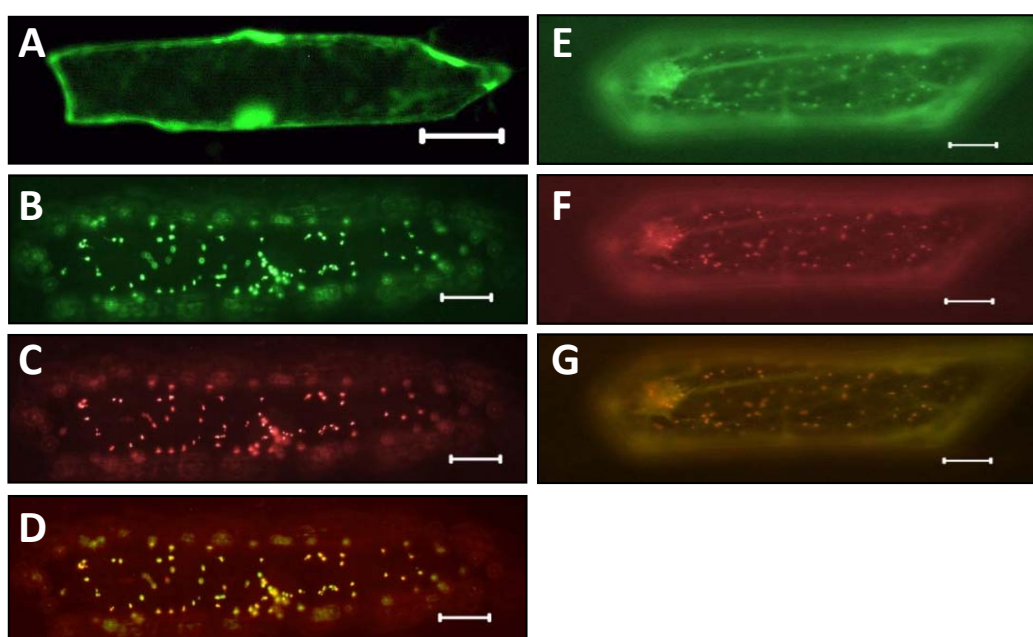


Fig. 2, Kihara *et al.*
Color print

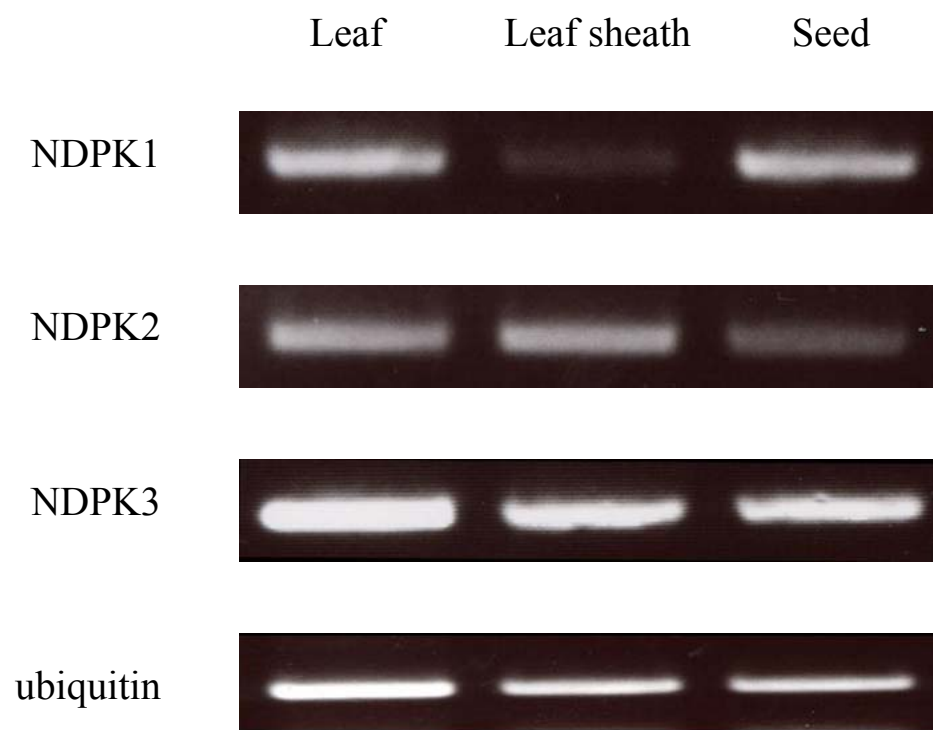


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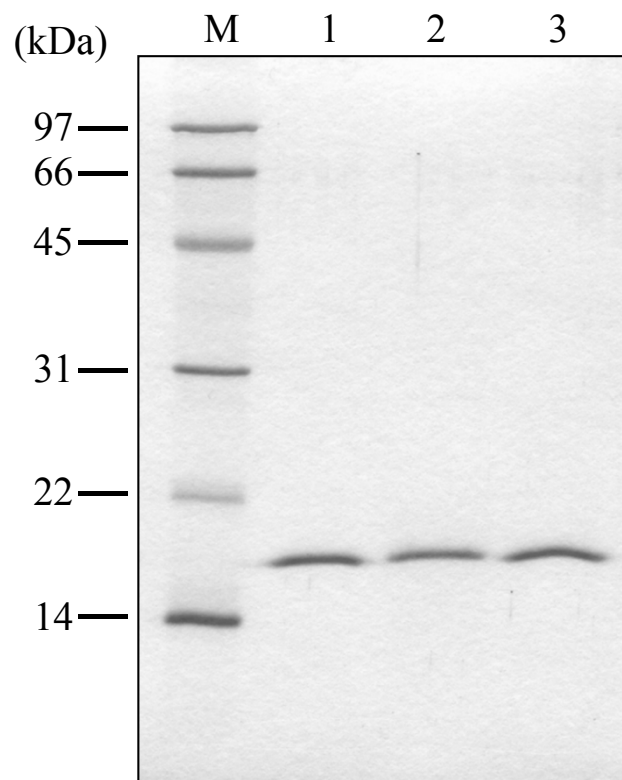


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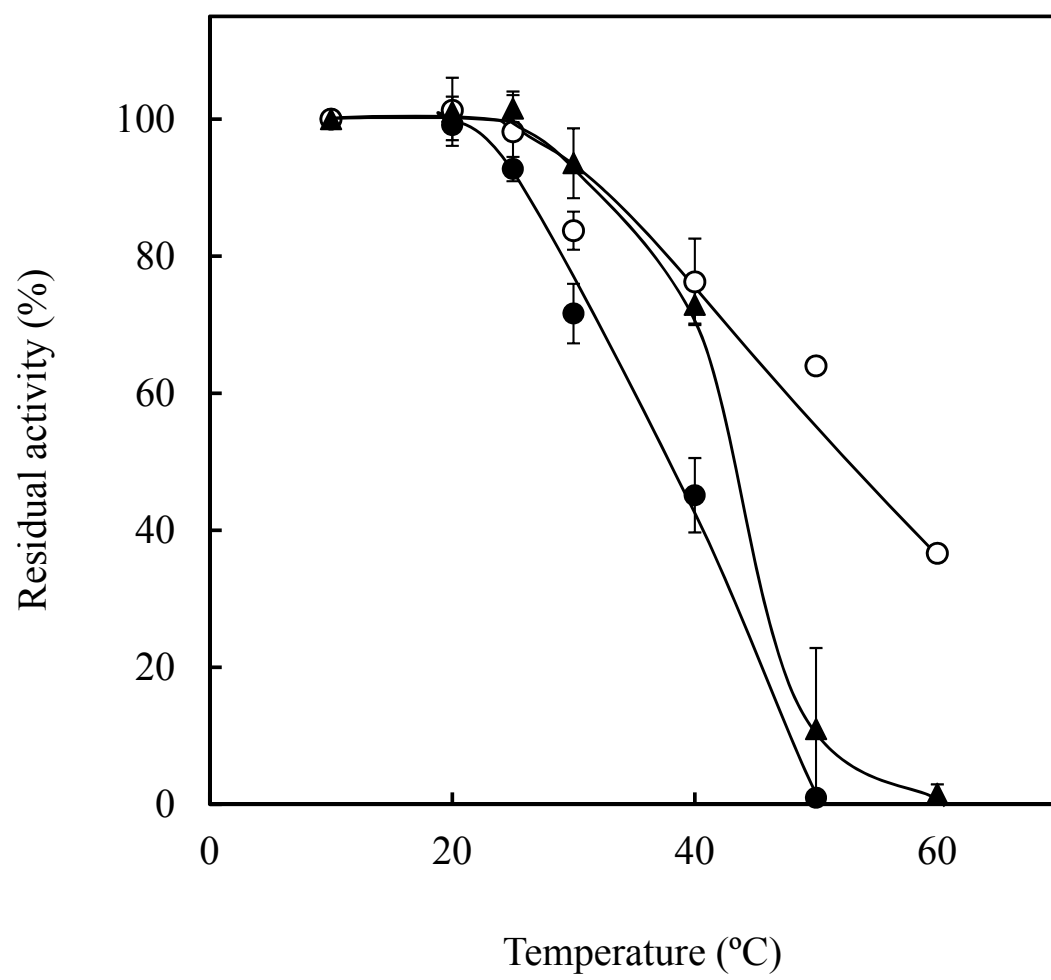


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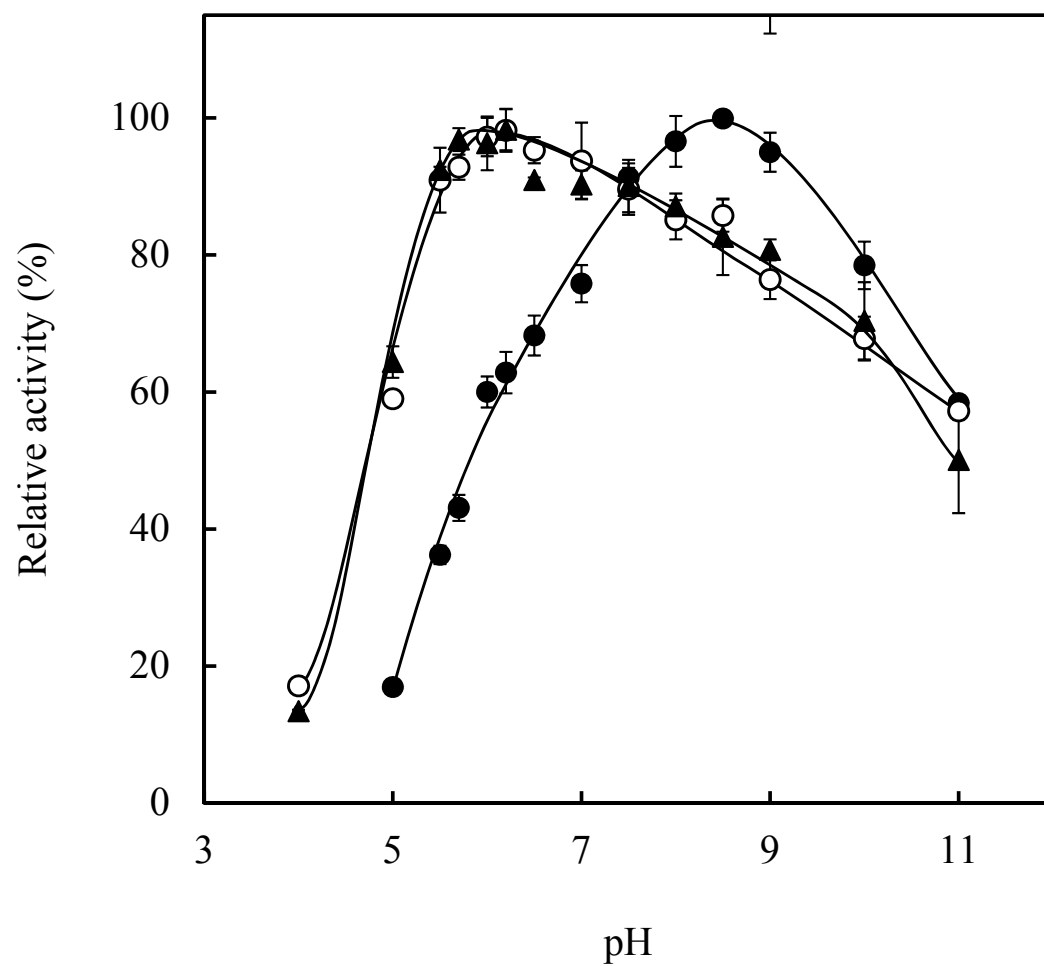


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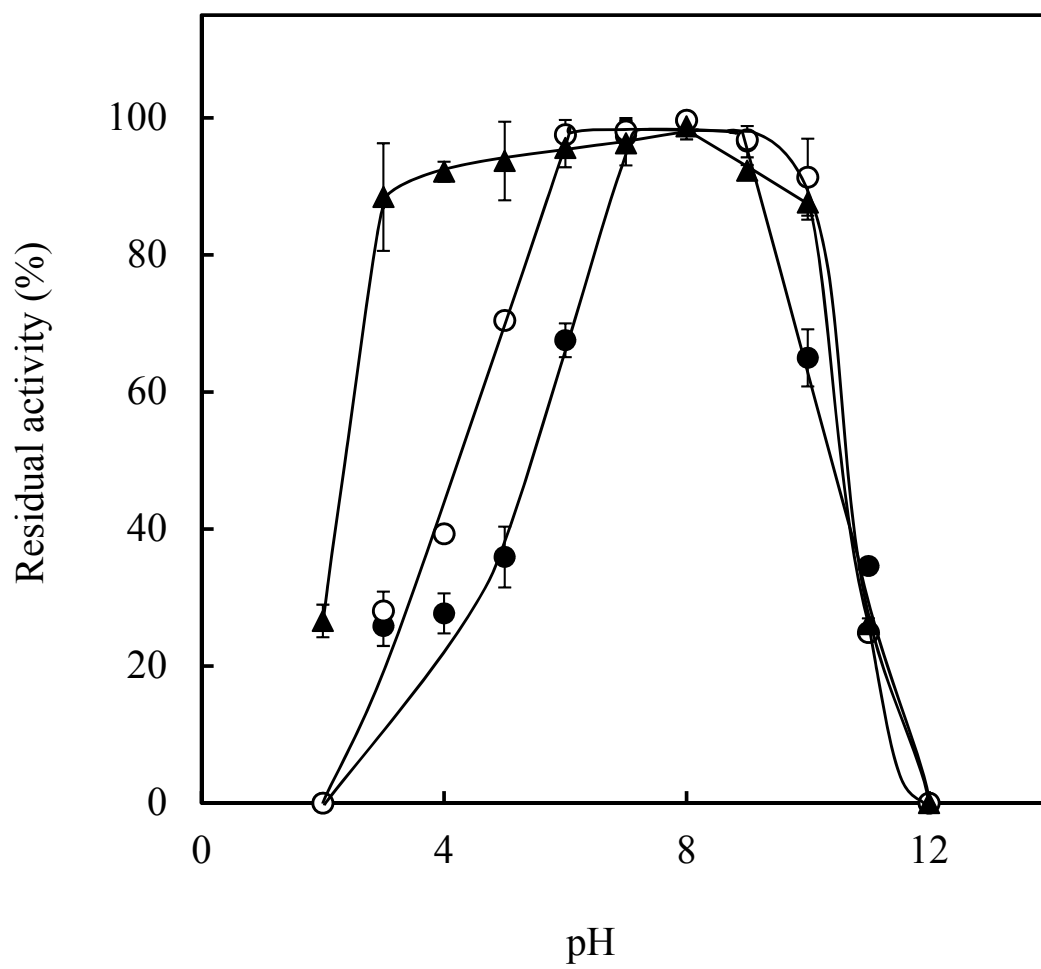


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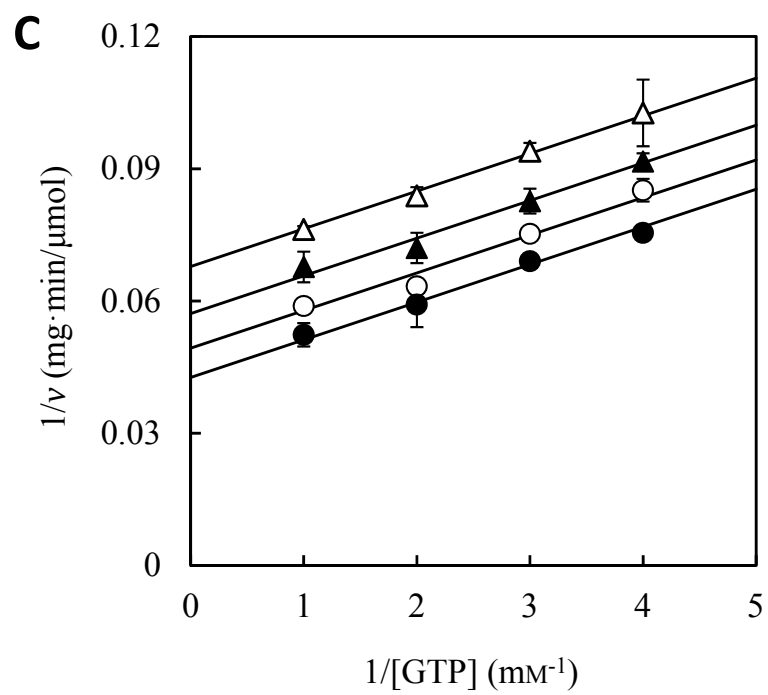
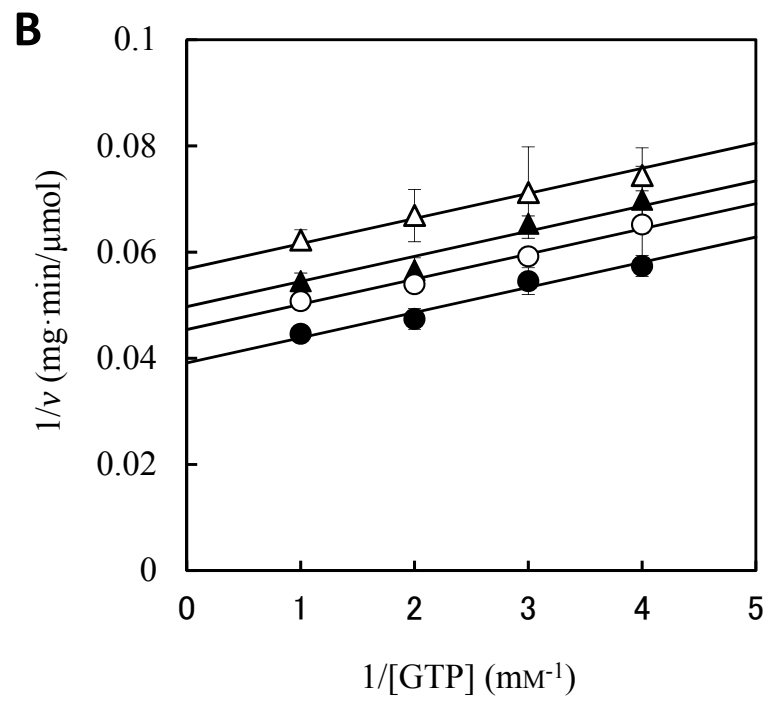
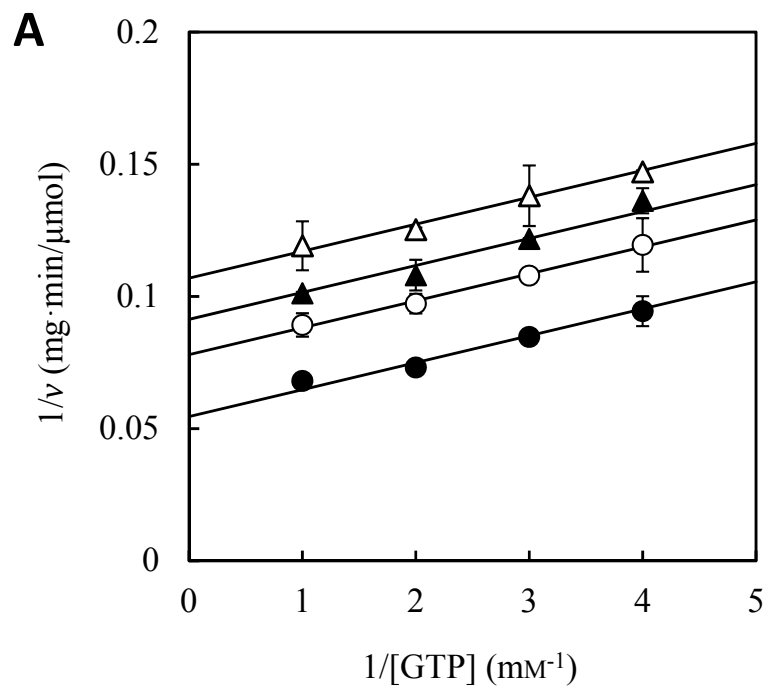


Fig. 8, Kihara *et al.*

Table 1. Kinetic Parameters of Recombinant OsNDPKs

(a) OsNDPK1

Substrate	k_{cat} (s ⁻¹)	K_{ma}^{a} (mM)	K_{mb}^{b} (mM)	$k_{\text{cat}}/K_{\text{ma}}$ (s ⁻¹ mM ⁻¹)	$k_{\text{cat}}/K_{\text{mb}}$ (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)	$k_{\text{cat}}/K_{\text{ma}}$ (s ⁻¹ mM ⁻¹)	$k_{\text{cat}}/K_{\text{mb}}$ (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)	$k_{\text{cat}}/K_{\text{ma}}$ (s ⁻¹ mM ⁻¹)	$k_{\text{cat}}/K_{\text{mb}}$ (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