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# *Arabidopsis* nucleoside diphosphate kinase-2 as a plant GTPase activating protein

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**Nucleoside diphosphate kinase (NDPK) is involved in multiple signaling pathways in mammalian systems, including G-protein signaling. *Arabidopsis* NDPK2, like its mammalian counterparts, is multifunctional despite its initial discovery phytochrome-interacting protein. This similarity raises the possibility that NDPK2 may play a role in G-protein signaling in plants. In the present study, we explore the potential relationship between NDPK2 and the small G proteins, Pra2 and Pra3, as well as the heterotrimeric G protein, GPA1. We report a physical interaction between NDPK2 and these small G proteins, and demonstrate that NDPK2 can stimulate their GTPase activities. Our results suggest that NDPK2 acts as a GTPase-activating protein for small G proteins in plants. We propose that NDPK2 might be a missing link between the phytochrome-mediated light signaling and G protein-mediated signaling. [BMB reports 2008; 41(9): 645-650]**

## INTRODUCTION

Nucleoside diphosphate kinase (NDPK) catalyzes the transfer of the γ-phosphate from nucleoside triphosphates to nucleoside diphosphates, using a ping-pong mechanism (1). NDPK is capable of maintaining nucleotide levels in mammals, implying NDPK may be a critical component of G protein-mediated signaling (2-6). Mammalian NDPK is a multifunctional enzyme, involved in the regulation of growth and development (7-9). Although the roles of plant NDPK are still unclear (10), progress has been made in elucidating NDPK's biological functions in *Arabidopsis*. Among three identified NDPK isoforms, NDPK2 interacts specifically with phytochrome in yeast two-hybrid screens (11) as well as in *in vitro* binding assays (12). Phytochrome apparently recognizes the hexameric structure of NDPK2 and stimulates its γ-phosphate exchange activity by modulating its active histidine residue (12, 13). Similar to mammalian NDPK, NDPK2 in plants possesses multiple functions, participating in the UV response (14), protection against reactive oxygen species stress (15), and auxin signaling (16).

Plant G proteins regulate many aspects of development and cell signaling, similar to their mammalian orthologs (17-19). In particular, both heterotrimeric and small G proteins contribute to light signal transduction (20-24). In oat, the Pr form of phytochrome enhances the GTPase activity of a small Ras-like G protein (24). In pea, mRNA levels of small Ras-like G proteins, including Pra2 and Pra3, are reversibly regulated by red and far-red light (25). Pra2 has been especially well characterized (26, 27). A recent study found that Pra2 regulates the cytochrome 450 that catalyzes C-2 hydroxylation in brassinosteroid biosynthesis, and this catalytic activation is down-regulated by light (28). Interestingly, the *Arabidopsis* orthologs of Pra2 and Pra3 have not been identified (unpublished data). In *Arabidopsis*, the heterotrimeric G proteins are directly involved in phytochrome-controlled seedling photomorphogenesis. To date, one α subunit (29, 30), one β subunit (31), and two γ subunits (32, 33) of the heterotrimeric G proteins have been identified in *Arabidopsis* (20, 34-41). Overexpression of GPA1, the only α subunit in *Arabidopsis*, resulted in a hypersensitive response to light, including a phytochrome-mediated inhibition of hypocotyl elongation (20). However, further evaluation of GPA1 using a loss-of-function approach challenged the direct role of heterotrimeric G proteins in phytochrome signaling (42). Therefore, the biological connection between heterotrimeric G proteins and phytochrome needs to be further examined. Due to the special role of NDPK in G protein-mediated signaling and the fact that NDPK2 was identified as a phytochrome-interacting protein, it will be important to see whether NDPK2 interacts with plant G proteins and consequently connects these two signaling pathways.

As an exploratory study, we report here on the interactions between NDPK2 and several selected plant G proteins *in vitro*. Our results indicate that NDPK2 interacts with plant small G-proteins and stimulate their GTPase activities. We propose that NDPK2 may function as a GTPase Activating Protein (GAP) for small G proteins in plants.

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## RESULTS

### NDPK2 interacts with plant small G proteins *in vitro*

To test the hypothesis that NDPK2 interacts with plant G proteins, an *in vitro* binding assay between NDPK2 and selected plant G proteins was conducted, using the small G proteins, Pra2 and Pra3, and the heterotrimeic G protein α subunit, GPA1. NDPK2 interacted strongly with both Pra2 and Pra3 (Fig. 1). In contrast, NDPK2 did not interact with GPA1 under the same conditions, either with or without the addition of GTP (Fig. 1). In addition, we also used a rice small G protein, Rgp1, as a negative control, and found no interaction between NDPK2 and Rgp1 (data not shown).

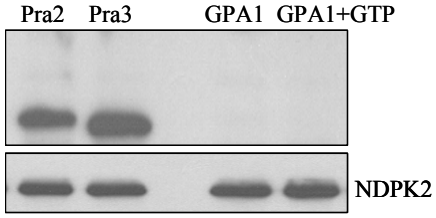


**Fig. 1.** NDPK2 interacts with plant small G proteins *in vitro*. *In vitro* binding assays were performed between NDPK2 and Pra2, Pra3, and the heterotrimeric G protein α subunit, GPA1. A specific antibody against NDPK2 was used to immunoprecipitate NDPK2. A monoclonal antibody against 6xHis tag was used to detect G proteins with a 6xHis fusion.

### NDPK2 functions as a GTPase activating protein

To investigate the functional relationship between NDPK2 and plant G proteins, the GTPase activities of Pra2, Pra3, and GPA1 were examined in the presence of increasing amounts of NDPK2. NDPK2 stimulated the GTPase activities of Pra2 and Pra3 significantly, whereas the GTPase activity of GPA1 was not affected by the presence of NDPK2 (Fig. 2A). In addition, a GTP binding assay of these G proteins was performed using GTPγS, a non-hydrolysable analog of GTP. NDPK2 addition affected GTPγS binding to Pra2 and Pra3 (Fig. 2B), but not to GPA1. Our data suggest that NDPK2 is able to interact with small G proteins *in vitro* and functions as a GTPase activating protein (GAP).

### The interaction between NDPK2 and small G proteins requires the hexameric structure of NDPK2

*Arabidopsis* NDPKs form hexameric structures (13) that are required for the *in vitro* interaction of NDPK2 with phytochrome (12). To investigate the role oligomeric states may play in the interaction of NDPK2 with small G proteins, we compared the *in vitro* binding activities of wild-type NDPK2 with a mutated form, LP175S, that is dimeric (12). *Arabidopsis* NDPK1 was also included as a hexameric negative control. Compared to the strong interaction between wild-type NDPK2 and small G proteins, no interaction was observed with the dimeric form, LP175S (Fig. 3A). In addition, the dimeric form of NDPK2 was also unable to enhance the GTPase activities of both Pra2 (Fig. 3B) and Pra3 (Fig. 3C).

LP175S contains two separate mutations: a C-terminal deletion (L225Stop) and a Kpn loop mutation (P175S). To determine if the lack of interaction between LP175S and small G proteins is due directly to the mutation (P175S) or the deletion (L225Stop), we also examined the binding activity of proteins containing only one or the other of these mutations. The C-terminal deletion mutant possess a higher γ-phosphate exchange activity than that of wild type, and P175S forms an unstable hexamer due to a Kpn loop mutation (12). We found no difference in the interaction with small G proteins of wild-type and the L225 mutated form of NDPK2 (Fig. 3B & 3C). The P175S

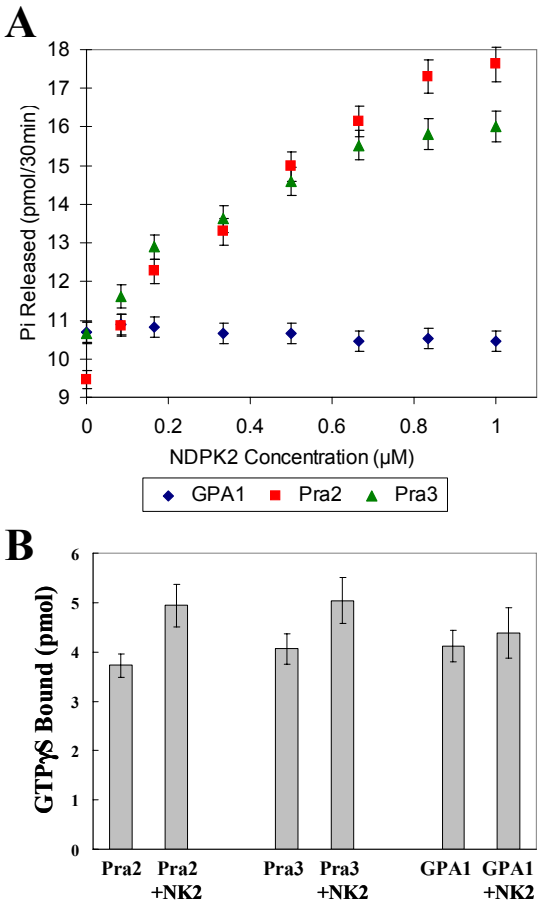


**Fig. 2.** NDPK2 functions as a GAP to plant small G proteins. (A) NDPK2 stimulates the GTPase activities of plant small G proteins. The GTPase activities of Pra2, Pra3, and GPA1 were measured with the addition of NDPK2. NDPK2 stimulated the GTPase activities of Pra2 and Pra3 significantly, but not that of GPA1. (B) The GTP binding ability of plant G proteins were examined with or without NDPK2 addition. A non-hydrolysable GTP analogue GTPγS was used as described.

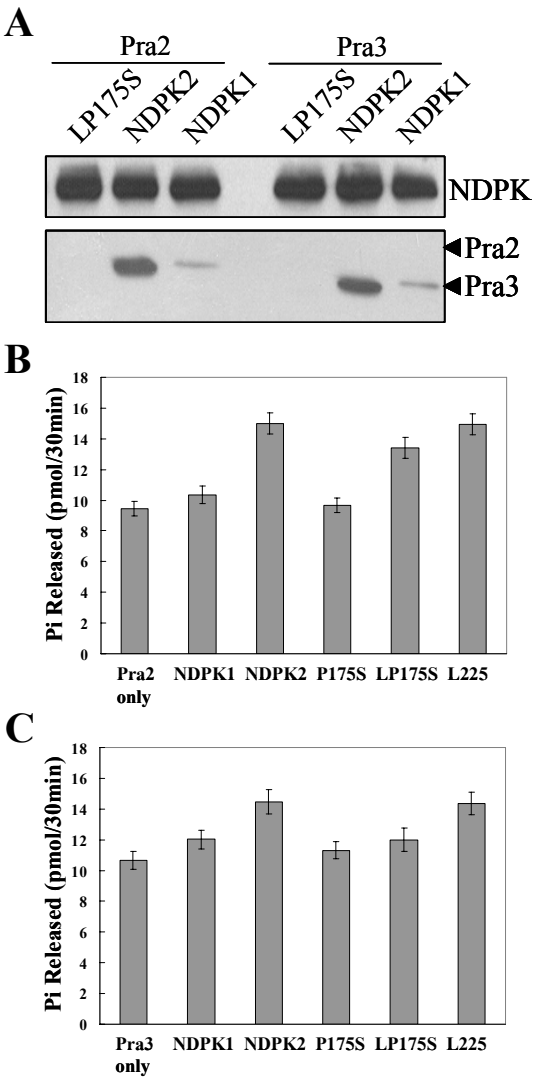


**Fig. 3.** NDPK2 requires its hexameric structure to interact with small G proteins. (A) *In vitro* binding assays between small G proteins and NDPKs, including NDPK1 (NK1), NDPK2 (NK2), and the dimeric form of NDPK2 (LP175S). Left panel, interaction with Pra2; Right panel, interaction with Pra3. (B) The oligomeric state of NDPK2 is important for NDPK2 GAP function. The GTPase activity of Pra2 was measured in the presence of different NDPKs and NDPK2 oligomeric state mutants. (C) The GTPase activity of Pra3 was measured in the presence of different NDPKs and NDPK2 oligomeric state mutants. NDPK1 was included as a negative control in both Pra2 and Pra3 experiments. P175S, NDPK2 mutant with a mutation in the Kpn loop; L225, NDPK2 deletion mutant L225Stop.

mutation caused a slight reduction in the interaction with small G proteins (Fig. 3B & 3C). These data suggest that the interaction between NDPK2 and small G proteins requires the hexameric form of NDPK2. Interestingly, NDPK1 showed a very weak interaction with both Pra2 and Pra3 (Fig. 3A) as well as a

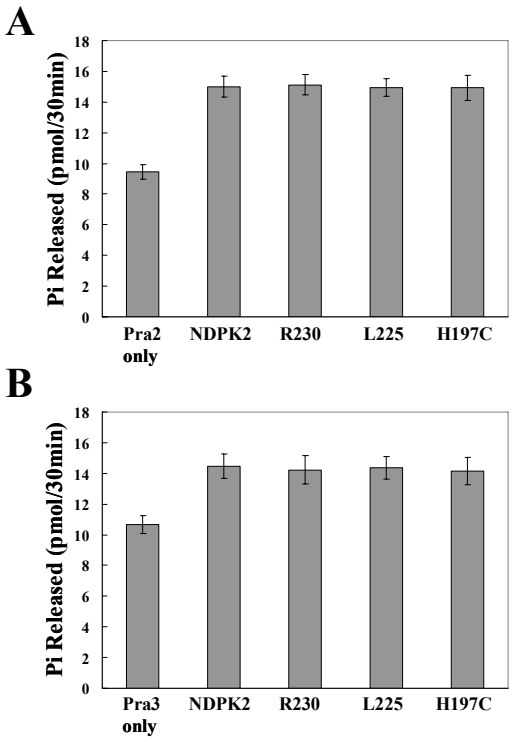


**Fig. 4.** The enzymatic activity of NDPK2 is separate from NDPK2 GAP function. (A) The GTPase activity of Pra2 was measured in the presence of NDPK2 mutants, including NDPK2 C-terminal deletion mutants and NDPK2 kinase site mutant, H197C. (B) The GTPase activity of Pra3 was measured in the presence of NDPK2 mutants, including NDPK2 C-terminal deletion mutants and NDPK2 kinase site mutant, H197C. R230, mutant R230Stop; L225, mutant L225Stop.

slight stimulation of their GTPase activities (Fig. 3B & 3C).

### The enzymatic activity of NDPK2 is separate from its GAP function

The NDPK2 C-terminal deletion mutants, R230Stop and L225Stop, possess a higher γ-phosphate exchange activity than that of wild type, whereas the NDPK2 kinase site mutant, H197C, has no enzymatic activity [12]. To investigate the role of NDPK2 enzymatic activity in its GAP function, the GTPase activities of both Pra2 (Fig. 4A) and Pra3 (Fig. 4B) were examined in the presence of the different NDPK2 mutants. The hyperactive NDPK2 mutants, R230Stop and L225Stop, did not stimulate Pra2 or Pra3 GTPase activities more than wild type (Fig. 4), suggesting that the enzymatic activity of NDPK2 is not directly involved in the interaction of NDPK2 with small G proteins. In addition, the kinase site mutant H197C was able to stimulate the GTPase activities of Pra2 and Pra3 similar to wild type (Fig. 4), confirming that the enzymatic activity of NDPK2 is not essential in this interaction. Taken together, these results suggest that the enzymatic activity of NDPK2 is

separate from its GAP function.

## DISCUSSION

Formation of GTP by NDPK contributes to the activation of G protein-mediated signaling. However, the mechanism by which NDPK interacts with G proteins remains relatively unknown, especially in plants. We explored the possibility that *Arabidopsis* NDPK2 interacts with plant G proteins, and established an *in vitro* interaction between NDPK2 and the plant small G proteins, Pra2 and Pra3. Our novel findings that *Arabidopsis* NDPK2 directly interacts with small G proteins to stimulate their GTPase activities suggest that NDPK2 is able to serve as a GAP for small G proteins in plants.

The oligomeric states of NDPK2 are critical for the protein-protein interaction of NDPK2 and its signaling partners, such as phytochrome. Our study of the *in vitro* interaction between NDPK2 and plant small G proteins further addressed this important feature of NDPK2. The dimeric mutant form of NDPK2 showed weaker binding to G proteins and GAP function when compared to wild type, which exists as a hexamer. Our characterization of the NDPK2 interaction with plant small G proteins suggests that the enzymatic activity of NDPK2 is separate from its GAP function. The kinase site mutant of NDPK2 is still capable of stimulating the GTPase activities of plant small G proteins, similar to wild type and the hyperactive NDPK2 mutants. These observations further confirmed the requirement of the NDPK2 hexameric structure in its protein-protein interactions.

The role of GPA1 in the phytochrome-mediated light signaling is controversial. As the sole α subunit of the heterotrimeric G protein complex in *Arabidopsis*, GPA1 is a potential target of NDPK2, but we found that NDPK2 is unable to interact with GPA1 and stimulate its GTPase activity *in vitro*. One possible interpretation could be that plant NDPK might not signal through the heterotrimeric G protein complex. Another possibility is that plant NDPK might interact with Gβγ subunits or other cofactors associated with GPA1 to conduct the signal. There is a specific interaction between the bovine transducin Gβ subunit and human NDPKB (Nm23-H2) (43). Therefore, plant Gβγ subunits may also interact with NDPK.

The GAP activity of NDPK2 reported here further extends our understanding of NDPK2 as a catalytic enzyme as well as a phytochrome signaling partner. NDPK2 is known as a positive signaling partner of phytochrome that interacts and stimulates the enzymatic activity of NDPK2 *in vitro* (11-13). The observed interaction between NDPK2 and small G proteins may potentially connect G protein-mediated signaling to phytochrome-mediated signaling through NDPK2. Even though Pra2 and Pra3 are from pea and their orthologs in *Arabidopsis* have not been identified, NDPK2 shares a high similarity with pea NDPKs and the phosphorylation of pea NDPKs is induced significantly by red light illumination (10). Thus, we can speculate that the G protein-mediated signaling could be linked to the phytochrome-mediated signaling *via* a specific NDPK isoform.

## MATERIALS AND METHODS

### Constructs for protein expression

Wild type and several altered forms of *Arabidopsis* NDPK2 were cloned into a pGEX-4T vector (Amersham, Piscataway, NJ) as described (12), including the C-terminal deletion mutants (R230Stop and L225Stop), the kinase site mutant (H197C), and two oligomeric state mutants (P175S and LP175S). *Arabidopsis* GPA1 was cloned into the pQE30 vector (Qiagen, Chatsworth, CA) with 5′ primer (*Sac*I): 5′-CGGAGCT-CGGCTTACTCTGCA GTAGAAG-3′ and 3′ primer (*Sma*I): 5′-CTCCCCGGGTAAA-A GGCCCAGCCTCCAG-3′. Pea Pra2 and Pra3 were cloned into the pQE30 vector using BamHI and SmaI restriction sites and prepared as previously described (29).

### *In vitro* binding assay

The *in vitro* binding assay between NDPK2 and G proteins was performed in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5 mM $MgCl_2$, 0.1% NP40 and protease inhibitors as described (12). Ten μg of NDPK2 and G proteins were co-immunoprecipitated using a specific antibody against NDPK2. In the binding assay testing NDPK2 specificity, a monoclonal antibody against the GST tag (Oncogene, Boston, MA) was used to immunoprecipitate the GST-NDPK proteins. After separation on SDS-PAGE (15%, w/v), protein samples were transferred to a polyvinylidene difluoride (PVDF) membranes for western blot analysis. Antibodies against NDPK2, GST and 6xHis tag (Qiagen, Chatsworth, CA) were used.

### Measurement of GTPase activity

Determination of GTPase activities of plant G proteins, including Pra2, Pra3, and GPA1, was performed in a 100 μL reaction mixture containing 8 pM G protein, 10 mM HEPES (pH 7.8), 5 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.05% Lubrol, and 2 μM GTP. The reaction was initiated by adding 5 μCi of $\gamma$-$^{32}$P-labeled GTP (Amersham, Piscataway, NJ) and incubated for 30 min at 30°C. The reaction was quenched by addition of 0.9 mL 5% (w/v) charcoal solution in 50 mM $NaH_2PO_4$. After centrifugation for 10 min, 0.5 mL of the supernatant was analyzed for the labeled free phosphate using a liquid scintillation counter. The effect of NDPK was examined by adding the indicated amount of NDPK proteins to the G protein reaction mixture. Mean values and standard deviation were calculated from three replicates.

### GTPγS binding assay

Binding of GTPγS to plant G proteins was determined by the rapid filtration method (44). Ten pmol of G protein was incubated for 30 min at 30°C in 200 μl of 50 mM HEPES buffer (pH 7.8), containing 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, and 1 μCi of [$\gamma$-$^{35}$S]GTP (Amersham, Piscataway, NJ) with or without 5 pmol of NDPK2. NDPK2 was pre-saturated with 1

μM dCDP before addition to G protein reaction mixture. The reaction was stopped by addition of 2 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl and 25 mM $MgCl_2$, followed by rapid filtration on 0.45 μm nitrocellulose filters. The filter-bound material was washed 4 times with the same ice-cold buffer, dried, and quantified for radioactivity by a liquid scintillation counter. Mean values and standard deviation were calculated from three replicates.

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