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Nucleoside diphosphate kinase isoforms regulated by phytochrome A isolated from oat coleoptiles

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Nucleoside diphosphate kinase (NDPK) (EC 2.7.4.6), the enzyme transferring the phosphate residue from ATP to nucleoside diphosphates, is localized mainly in the cytoplasm and mitochondria and in smaller amounts in cell nuclei and the microsomal fraction. Exposure of etiolated oat seedlings to red light causes an increase of the enzyme activity by about 42% in nuclear fraction, 7% in etioplastic and 14% in postetioplastic fraction. Endogenous phytochrome A, as visualized by an immunochemical method, translocates from the cytoplasm into the nucleus upon red, far-red or white light activation. Nuclei purified from oat seedlings contain two, and the postnuclear fraction four easily separated forms of NDPK. One of the nuclear isoforms (I_n) and one isoform isolated from the postnuclear fraction (II_{pn}) are activated by red light in the presence of phytochrome A purified from etiolated oat coleoptiles. Both phytochrome A-activated NDPKs purified to electrophoretic homogeneity have the same molecular mass (17–18 kDa) determined by SDS/PAGE. Both enzymes in the native form have similar molecular masses (71 and 63 kDa).

Keywords: NDPK (nucleoside diphosphate kinase), phytochrome A, NDPK isoforms, phytochrome A nuclear import

INTRODUCTION

Nucleoside diphosphate kinase (NDPK) (EC 2.7.4.6) is a phosphotransferase transferring the phosphate residue from nucleoside triphosphates (NTP), mainly ATP, to 5′-nucleoside diphosphates (NDP) according to the following equation:

$$ATP + E \Leftrightarrow E\text{-}P + ADP$$
$$E\text{-}P + NDP \Rightarrow E + NTP$$

NDPK, discovered over 50 years ago in yeast and in pigeon muscle cells (Parks & Agarwal, 1973; Lacombe *et al*., 2000), plays a key role in the maintenance of physiological concentrations of ribo- and deoxyribonucleotide triphosphates in the cell. In recent years, interest in this enzyme has increased especially after cloning of the *awd* gene from *Drosophila melanogaster* and the human *NM23* gene (Lacombe *et al*., 2000). The NDPK isoforms encoded by these genes fulfill functions totally different from those that were previously ascribed to the enzyme. The progress of investigation in the succeeding years has confirmed that some NDPK isoforms from animal tissues are involved in the regulation of transcription, others in cell differentiation and yet others play the role of phosphotransferases transferring the phosphate residue to proteins (Kikkawa *et al*., 1990; Bominaar *et al*., 1993; Kimura *et al*., 2000; 2003; Otero, 2000; Baillat *et al*., 2002; Narayanan & Ramaswami, 2003).

The results of investigations performed on plant material link the role of NDPK with plant responses to stress factors such as wounding (Harris *et*

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Abbreviations: ARR4, *Arabidopsis* response regulator4; Aux/IAA, early auxin gene; Cry1, cryptochrome 1; ELF, early flowering; HFR/REP1/RSF1, long hypocotyls in far-red/reduced phytochrome signaling 1/reduced sensitivity to far-red light; NDPK, nucleoside diphosphate kinase; NDP, nucleoside diphosphate; PIFs, phytochrome interacting factors; PKS1, phytochrome kinase substrate 1; Pr and Pfr, red and far-red absorbing forms of phytochrome, respectively.

al., 1994), thermal stress (Moisyadi *et al*., 1994; Leung & Hightower, 1997; Hurkman *et al*., 1998; Barthel & Walker, 1999; Escobar Galvis *et al*., 2001), oxidative stress (Moon *et al*., 2003; Yang *et al*., 2003) and UV-B (Zimmermann *et al*., 1999). Moreover, results of several experiments performed on pea have shown that red light activates NDPK phosphorylation (Tanaka *et al*., 1998). Research using the yeast two-hybrid system has confirmed the physical interaction of recombinant phytochrome A with one of the NDPK isoforms from *Arabidopsis thaliana*, suggesting that the NDPK2 isoform functions in the phytochrome signal transduction pathways (Choi *et al*., 1999). Results of the most recent investigations indicate that NDPK2 also participates in auxin signaling playing a role in phytohormone transport (Choi *et al*., 2005), and cytosolic NDPK is involved in growth and cell division (Dorion *et al*., 2006), pea mitochondrial NDP kinase (NDPK3) cleaves DNA and RNA (Hammargren *et al*., 2007). A similar role is possible for NDPK2 localized in chloroplasts and supposedly associated with nucleoids (Bölter *et al*., 2007). The aim of our investigation was the separation of NDPK isoforms localized in the nucleus and the postnuclear fraction obtained from etiolated oat seedlings and identification and purification of isoforms subjected to regulation by red light *via* phytochrome A.

MATERIALS AND METHODS

Plant material. The plant material used in the investigations were 5-day seedlings of the oat variety Chwat, growing in the dark at 25°C.

Separation of subcellular fractions. Excised coleoptiles were homogenized manually in a chilled mortar inlaid with a nylon net in a homogenizing solution containing: 10 mM Tris/HCl buffer, pH 7.5, 0.3 M sucrose, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol (at a ratio of 6 ml solution per 1 g tissue). The homogenate was filtered through four layers of cheesecloth and fractionated by centrifugation. The crude nuclear fraction was obtained after 15 min of centrifugation at $700 \times g$. The obtained supernatant was centrifuged for 2 min at $6000 \times g$ in order to sediment the etioplastic fraction according to Evans and Smith (1976). The obtained supernatant, subsequently designated as the "postetioplastic" fraction, was centrifuged for 20 min at $20000 \times g$ in order to sediment the mitochondrial fraction, and the obtained supernatant was centrifuged in an ultracentrifuge at $60000 \times g$ for 60 min in order to sediment the microsomal fraction. The obtained supernatant was treated as the soluble protein fraction (cytosolic fraction).

Purification of cell nuclei. The crude nuclear fraction suspended in a small volume (1–10 ml) of homogenizing buffer was layered on a solution containing 0.3 M sucrose and 40% glycerol prepared in 10 mM Tris/HCl buffer, pH 7.5, containing 10 mM KCl, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol placed in centrifuge tubes. Centrifugation at $700 \times g$ for 15 min allowed the sedimentation of nuclei. The pellet of purified nuclei was suspended in homogenization medium or in 10 mM Tris/HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. The nuclear protein extract was obtained by sonication of the purified nuclei using an ultrasonic disintegrator (type UD-20 Techpan). Samples not exceeding 5 ml were sonicated on ice for 60 s (4×15 s) and subsequently centrifuged for 20 min at $20000 \times g$.

Light-regulated nuclear accumulation of phytochrome A. Five-day-old etiolated seedlings were exposed to red, far-red, blue or white light for 20 min and after additional 30 min dark treatment 5 g of plants was homogenized for nuclei isolation. To 1 ml nuclear protein extract obtained by sonication, 50 µl of polyclonal antibodies raised in rabbits against phytochrome A was added. After 30 min incubation in ice, protein A immobilized on 250 µm acrylic beads was used for immunoprecipitation. The pellet obtained after centrifugation was suspended in 100 mM glycine/HCl buffer, pH 2.5. Acrylic beads were centrifuged and the obtained supernatant was analysed by SDS/PAGE and immunobloting.

Immunobloting. SDS/PAGE was performed according to the method of Ogita and Markert (1979) in a Mini Protean II electrophoresis cell (Bio-Rad) using 12% (w/v) resolving gel. The molecular mass standard was the 10 kDa Protein Ladder (Gibco). The separated proteins were transferred to a nitrocellulose membrane in buffer containing 50 mM Tris, 380 mM glycine, 0.1% (w/w) SDS and 20% (v/v) methanol. Positions of the protein markers were visualized by staining with Ponceau S. The blot was incubated with primary antibodies against phytochrome A purified from oat coleoptiles. The position of phytochrome was detected using goat anti-rabbit-IgG antibodies (Sigma) conjugated to alkaline phosphatase (Harlow & Lane, 1988).

Red, far-red or blue light irradiation of coleoptiles. Five-day-old etiolated seedlings were exposed to red, far-red or blue light for 20 min, and after an aditional 30 min dark treatment plants were homogenized for nuclei, etioplasts and postetioplastic fraction separation. The crude nuclear fraction was obtained after 15 min of centrifugation at $700 \times g$. Subsequently, the etioplastic fraction was obtained after 2 min of centrifugation at $6000 \times g$ as described by Evans and Smith (1976). The obtained supernatant was designated as the postetioplasic fraction.

Isolation and purification of NDPK. The nuclear protein extract was used to purify NDPK directly after it was obtained, whereas proteins con-

tained in the postnuclear fraction were salted out using ammonium sulphate to 67% saturation and were stored at 4°C until use.

Ion exchange chromatography on DEAE-Sephacel. Nuclear proteins were applied to a column (2.5 cm×10 cm) filled with DEAE-Sephacel, equilibrated with 25 mM Tris/HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol (buffer A). Proteins not bound to the carrier were eluted with buffer A until the absorbance of the eluate at 280 nm was below 0.1. Proteins bound to the bed were eluted with a linearly increasing chloride ion concentration in buffer A (from 0 to 300 mM NaCl) at 0.8 ml/min. Fractions of 6 ml were collected.

Proteins of the postnuclear fraction salted out with ammonium sulfate to 67% saturation were centrifuged and subsequently dialyzed overnight against buffer A. The clear supernatant obtained after centrifugation of the denatured proteins was applied to a DEAE-Sephacel column equilibrated with buffer A. Proteins not bound to the column were eluted with buffer A, and those bound to DEAE-Sephacel were eluted as above. Proteins remaining on the column were eluted with buffer A containing 300 mM NaCl. Fractions with the highest NDPK activity were pooled and concentrated to 1–3 ml in an Amicon 8050 filtration apparatus (Diaflo YM 10 membrane).

Chromatography on Bio-Gel HTP hydroxyapatite. The NDPK preparation obtained after ion exchange chromatography was applied at 0.4 ml/min to a hydroxyapatite column (1.5 cm×5 cm) equilibrated with buffer A. Proteins not bound to the bed were eluted with buffer A until the absorbance at 280 nm was less than 0.05, whereas proteins remaining on the column were eluted with a linearly increasing phosphate ion concentration (50 ml of buffer A and 50 ml 400 mM (K) phosphate buffer, pH 7.5). Fractions (6 ml) were collected. The fractions with the highest enzyme activity were pooled and concentrated under nitrogen to about 1 ml.

Desalting of the enzyme on Sephadex G-10 gel. The concentrated enzyme preparation obtained after adsorption chromatography was applied to a 1.5 cm×15 cm Sephadex G-10 column in order to remove phosphates. The proteins were eluted with buffer A, and fractions having NDPK activity were pooled and concentrated to of about 1 ml.

Chromatography on GTP-agarose. The enzyme preparation depleted of phosphate ions was applied to a minicolumn containing 1 ml GTP-Agarose equilibrated with 25 mM Tris/HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol and 1 mM $MgCl_2$ (buffer B). Proteins not bound to the column were eluted with buffer B, whereas proteins remaining on the bed were eluted with 25 mM Tris/HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 25 mM NaCl and 1 mM ATP (buffer C). Three active fractions of 2 ml each were concentrated to about 0.5 ml.

Determination of molecular mass of NDPK by molecular filtration on Sephadex G-150. A 2.5 cm×86 cm Sephadex G-150 column equilibrated with buffer A was calibrated using the following protein standards: myoglobin (17.6 kDa), chicken egg albumin (45 kDa), bovine serum albumin (67 kDa) and hexokinase (95 kDa). Isoforms of NDPK of the nuclear fraction and the postetioplastic fraction obtained after ion exchange chromatography were then separated on the calibrated column. Proteins were eluted with buffer A at 0.07 ml/min. Fractions of 1.4 ml were collected and used for NDPK activity assays.

Determination of molecular mass of NDPK isoforms by denaturing electrophoresis. Electrophoresis under denaturing conditions was performed according to Ogita and Markert (1979) using 4% (w/v) stacking gel and 16% (w/v) separating gel. A mixture of polypeptides (Protein Leadder) obtained from Gibco was used as standards.

Assay of nucleoside diphosphate kinase activity. The method is based on assaying the ADP formed in reaction 1, which in the presence of phosphoenolpyruvate is phosphorylated to ATP by phosphoenolpyruvate kinase (reaction 2), and the pyruvate formed in this reaction is reduced in the presence of NADH by lactate dehydrogenase (reaction 3)

$$ATP + dCDP \Leftrightarrow ADP + dCTP \quad \text{(reaction 1)},$$

$$ADP + \text{phosphoenolpyrvate} + H^+ \Leftrightarrow ATP + \text{pyruvate} \quad \text{(reaction 2)},$$

$$\text{pyruvate} + NADH + H^+ \Leftrightarrow \text{L-lactate} + NAD^+ \quad \text{(reaction 3)}.$$

The reaction medium (1 ml) contains: 50 mM Hepes/KOH buffer, pH 7.5, 25 mM KCl, 6.25 mM $MgCl_2$, 2 mM ATP, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 1.95 units of pyruvate kinase and 3.75 units of lactate dehydrogenase. After adding the analyzed tissue extract or the NDPK preparation, the hydrolytic activity in respect to ATP was first determined and subsequently 0.5 μmole of dCDP was added (acceptor of the phosphate residue from ATP, not subject to phosphorylation by pyruvate kinase). The velocity of oxidation of reduced $NADH_2$ in the reaction medium measured spectrophotometrically at 340 nm is a measure of the velocity of the reaction. The assays were performed at room temperature (Shimadzu UV-Visible Recording Spectrophotometer UV-160A).

Analysis of *in vitro* effects of phytochrome A on NDPK activity. Phytochrome A was isolated and purified from etiolated oat coleoptiles according

to Vierstra and Quail (1983). The obtained phytochrome A did not contain NDPK and did not show a hydrolytic activity towards ATP. The effect of phytochrome A on NDPK activity was analyzed in a standard medium used for assays of NDPK to which 50 μl of a solution containing purified phytochrome A was added. After determining the initial velocity of the reaction, the cuvette with the analyzed sample was removed from the spectrophotometer, illuminated for 5 min with red or far red light using a projector with interference filters, and the decrease in absorbance at 340 nm was measured again. The effect of red and far-red light on the velocity of oxidation of reduced NADH in a medium without phytochrome A was also checked.

RESULTS AND DISCUSSION

Subcellular localization of NDPK in etiolated oat seedlings

Total NDPK activity determined in crude homogenates obtained from etiolated oat seedlings was within the range of 28.9 to 31.5 μmol dCTP/min, and after fractionation of subcellular structures, the total activity of all fractions increased to 31.8–37.5 μmol dCTP/min (increase by about 5–24%). The results of experiments presented in Table 1 and obtained from three repeats indicate that approx. 81–85% of total NDPK activity occurred in the cytoplasmic fraction, 14–17% in the mitochondrial + etioplastic fraction, 0.5–1.4% in the nuclear fraction and about 1% of the total activity of the analyzed enzyme was associated with the microsomal fraction.

Effects of the red, far-red or blue-light irradiation of coleoptiles on NDPK activity

The results of experiments presented in Table 2 show that in the nuclear fraction obtained from red-light irradiated coleoptiles, NDPK activity (0.3 μmol dCTP/min per 10 g fresh weight) increased by about 42% as compared with the total enzyme activity in the nuclear fraction isolated from coleoptiles grown in darkness (0.2 μmol dCTP/min per 10 g fresh weight). The NDPK activity in the etioplastic and postetioplastic fractions isolated from red-light irradiated coleoptiles increased by about 7% and 14%, respectively. The enzyme activity in the same subcellular fractions isolated from far-red or blue-light irradiated coleoptiles was nearly identical to that in non-irradiated plants (Table 2).

Table 1. Subcellular localization of NDPK in etiolated oat seedlings.

Values presented in the table are the result of three repeats.

Subcellular fraction	Total activity (μmole dCTP/min)	Fraction of total activity (%)
Nuclear	0.2–0.5	0.5–1.4
Mitochondrial + etioplastic	4.4–6.3	13.8–16.7
Microsomal	0.2–0.3	0.7–0.8
Cytoplasmic	27.0–30.4	81.0–84.9

Light-regulated nuclear import of phytochrome A in oat coleoptiles

Light-induced nuclear import of phytochrome A was studied by immunoprecipitation using polyclonal antibodies against oat phytochrome A. Etiolated oat coleoptiles exposed for 20 min to red, far-red, blue or white light were excised after a subsequent 30 min dark period. Protein extracts obtained from isolated nuclear fraction were incubated with antibodies, and the imunocomplexes formed were immunoprecipitated on protein A immobilized on acrylic beads. Pellets separated by centrifugation were dissolved in acidic buffer and after SDS/PAGE separation of proteins, phytochrome A was analysed by immunoblotting. Results shown in Fig. 1. demonstrate that nuclear accumulation of phytochrome A occurs only under red (lane 3), far-red (lane 4), or white light (lane 6) irradiation. The immunoprecipitated phytochrome A is by about 10 kDa smaller than purified phytochrome A preparation (lane 1). In contrast, nuclear fraction isolated from dark-grown

Table 2. Activation of NDPK by red, far red and blue light illumination of oat coleoptiles

Subcellular fraction	Total activity (μmole dCTP/min per 10 g fresh weight)					
	Dark control	15 min red + 30 min dark	Dark control	15 min far red + 30 min dark	Dark control	15 min blue + 30 min dark
Nuclear	0.2 ± 0.0	0.3 ± 0.1 (142%)	0.6 ± 0.1	0.6 ± 0.1 (100%)	0.6 ± 0.1	0.5 ± 0.0 (82%)
Etioplastic	2.7 ± 0.5	2.9 ± 1.0 (107%)	2.8 ± 0.6	2.6 ± 0.3 (93%)	2.7 ± 0.3	2.6 ± 0.3 (99%)
Postetioplastic	22.9 ± 2.6	26.1 ± 2.0 (114%)	15.8 ± 3.1	17.4 ± 0.0 (110%)	16.7 ± 2.0	17.0 ± 1.5 (101%)

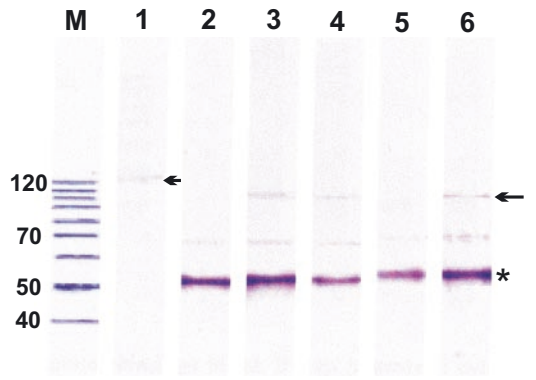

Figure 1. Light-regulated nuclear accumulation of phytochrome A.
Immunoblot analysis of immunocomplexes precipitated on protein A immobilized on acrylic beads. Lane M, molecular mass markers; lane 1, purified phytochrome A; lane 2, coleoptiles from darkness; lane 3, red-light; lane 4, far-red light; lane 5, blue light; lane 6, white light treated coleoptiles. Arrows mark phytochrome A, asterisk marks IgG heavy chain.

(lane 2) or blue light irradiated plants (lane 5) does not contain detectable amounts of phytochrome A.

Isolation of NDPK isoforms regulated by light

Ion exchange chromatography of proteins of the nuclear fraction

The NDPK elution profile presented in Fig. 2 indicates that cell nuclei of oat coleoptiles contain two isoforms of the enzyme which are easy to separate; the first, further referred to as I_n, elutes from the column at 30 mM chloride ion concentration, whereas the second isoform elutes from the bed only at 110 mM NaCl.

Ion exchange chromatography of proteins from the postnuclear fraction

The elution profile of NDPK shown that the postnuclear fraction contains NDPK isoforms eluted from the column in the form of well-separated peaks of enzymatic activity (Fig. 3). Peak I_{pn} is eluted by buffer A, peak II_{pn} is eluted by 30 mM [Cl^-], peak III_{pn} is released from the column with 110 mM chloride ions and peak IV_{pn} was only eluted by 300 mM NaCl.

Identification of NDPK isoforms regulated by phytochrome A

The two nuclear NDPK isoforms and the four isoforms from the postnuclear fraction obtained after DEAE-Sephacel chromatography were analyzed for their susceptibility to activation by phytochrome A. The activity of each NDPK isoform was assayed in a standard reaction medium in the presence of the same amount of photoreversible phytochrome A. In preliminary investigations, we established that phytochrome A purified from oat coleoptiles did not contain NDPK activity and did not hydrolyze ATP (not shown). After determining the initial activity, the cuvette with the analyzed sample was taken out of the spectrophotometer, irradiated with red light (480 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or far red light (24 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and the decrease of absorbance at 340 nm was measured again. In preliminary experiments, we established that irradiation of particular NDPK isoforms with red or far-red light in the absence of phytochrome A in the reaction medium did not affect the catalytic activity of the enzyme. The changes in NDPK activity observed as a result of irradiation of the samples with red or far-red light in the presence of phytochrome A are presented in Table 3. The obtained results indicate that the I_n nuclear isoform of NDPK is distinctly activated by red light in the presence of phytochrome A. The activity of the enzyme is increased over three-fold after 5-min irradiation of the sample. Photoconversion of phytochrome A to the Pr form after irradiation of the same sample with far-red light does not reverse the effect obtained with red light but leads to a further increase of the NDPK enzymatic activity by about 16%.

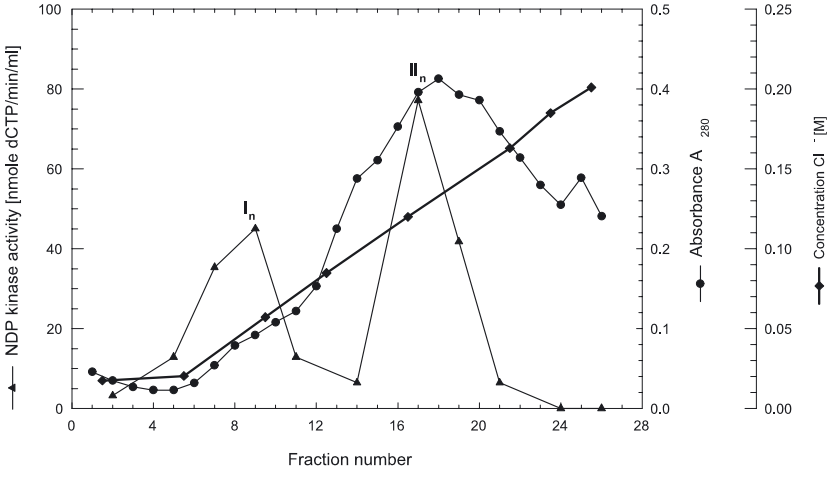

Figure 2. Chromatography on DEAE-Sephacel of nuclear NDPK isoforms.
The NaCl gradient was from 0 to 300 mM. Two NDPK isoforms were eluted at 30 mM (NDPK I_n) and 110 mM (NDPK II_n) Cl^- in the buffer, respectively.

Table 3. Changes in catalytic activity of NDPK isoforms accompanying phytochrome A photoconversion

Illumination	Activity of NDPK (nmole dCTP/10 min per 50 μl)					
	Nuclear isoforms		Postnuclear isoforms			
	I_n	II_n	I_{pn}	II_{pn}	III_{pn}	IV_{pn}
None	11.8±1.3 (100%)	30.3±2.5 (100%)	17.7±2.2 (100%)	24.1±3.1 (100%)	6.4±1.1 (100%)	19.3±2.3 (100%)
5 min red light	35.9±5.1 (304%)	29.4±2.1 (97%)	19.3±2.6 (109%)	32.2±4.3 (134%)	6.4±1.1 (100%)	19.3±2.3 (100%)
5 min red light/ 5 min far-red light	41.6±4.8 (353%)	28.5±1.8 (94%)	19.3±1.9 (109%)	32.2±3.8 (134%)	6.4±1.1 (100%)	19.3±2.3 (100%)

Analyzing the results shown in Table 3, it is worth noting that besides the nuclear I_n isoform, only isoform II_{pn} from the postnuclear fraction is clearly activated by red light in the presence of phytochrome A. The activity of this isoform increases after irradiation with red light by about 34% and does not change after photoconversion of phytochrome A to the Pr form. The activity of the other isoforms changed only slightly or not at all after irradiation with red or far-red light and in the presence of phytochrome A (Table 3).

Purification of NDPK isoforms I_n and II_{pn} regulated by light

The purification of NDPK isoforms I_n and II_{pn} essentially encompassed three steps. In the first, the previously described ion exchange chromatography on DEAE-Sephacel was used (Figs. 2 and 3). The concentrated isoform I_n and II_{pn} preparations obtained after ion exchange chromatography were separated using adsorption chromatography. Proteins not bound to hydroxyapatite were eluted with buffer A, whereas proteins adsorbed on the column were eluted with (K) phosphate buffer, pH 7.5, with a linearly increasing phosphate ion concentration from 0 to 400 mM. The results of the separation presented in Fig. 4A and 4C indicate that both I_n and II_{pn} isoforms bind relatively strongly to the carrier as they begin to be eluted from the column only at about 170 mM phosphate ion concentration. Samples containing NDPK activity were pooled and concentrated to about 0.5 ml, and after removal of phosphate ions on a Sephadex G-10 column, they were subjected to affinity chromatography on GTP-Agarose. Proteins not bound to the column were eluted with buffer A containing 1 mM $MgCl_2$ (buffer B), whereas proteins bound to the bed were eluted with 1 mM ATP in buffer C (Fig. 4B, D).

Electrophoresis in 16% polyacrylamide gel (SDS/PAGE)

In order to check the homogeneity of the obtained enzyme preparations and to determine the molecular mass of both NDPK isoforms, electrophoresis under denaturing conditions was performed on a 16% polyacrylamide gel (SDS/PAGE). In total, 0.87 μg of isoform I_n protein and 0.6 μg isoform II_{pn} protein were applied to the gel. The appearance of

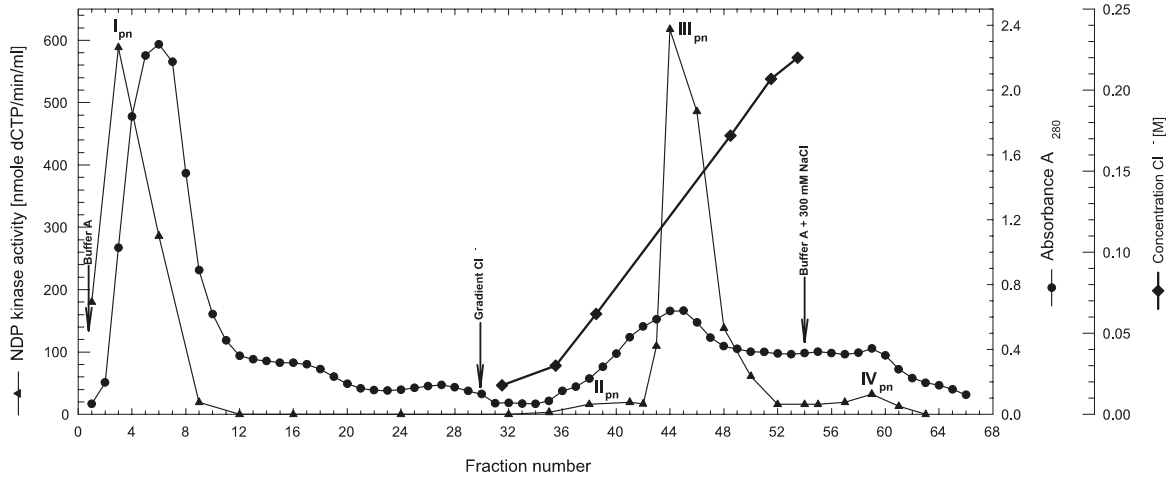

Figure 3. Elution profile of NDPK isoforms from postnuclear fraction.
The NaCl gradient was from 0 to 300 mM. Four NDPK isoforms were eluted succesively: NDPK I_{pn} with 25 mM Tris/HCl buffer, pH 7.5; NDPK II_{pn} at 30 mM Cl^-; NDPK III_{pn} at 110 mM; and NDPK IV_{pn} at 300 mM NaCl.

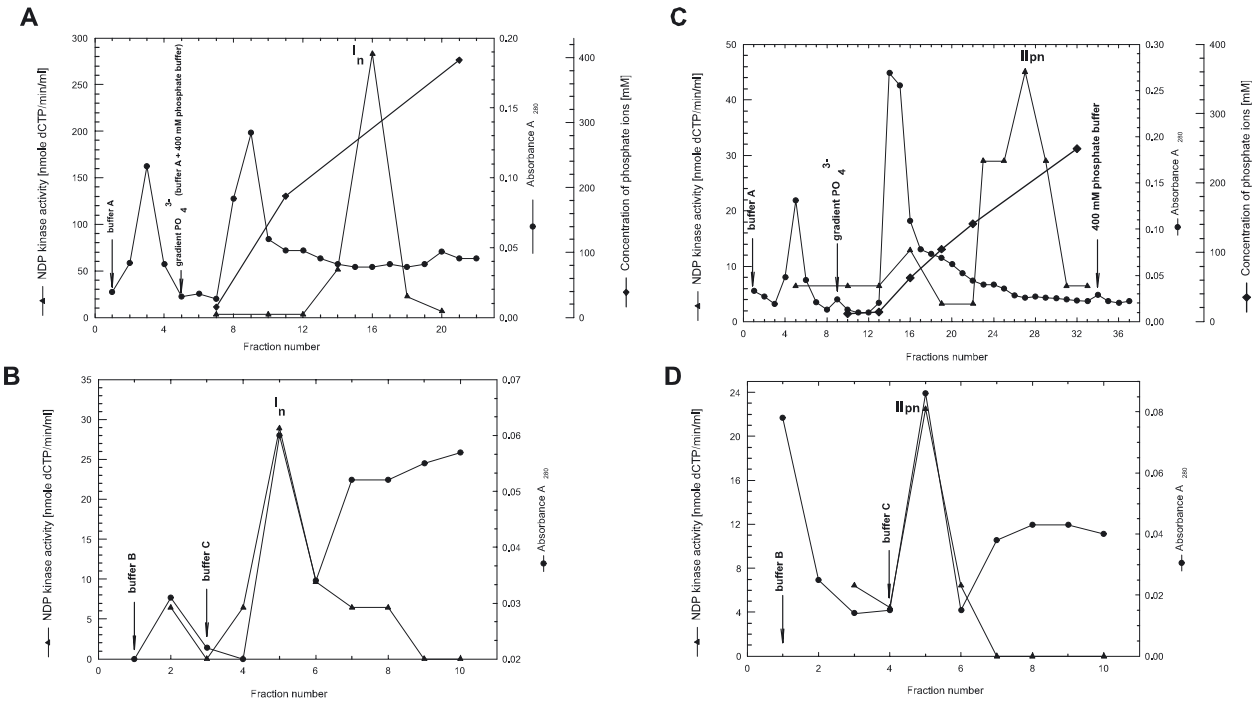

Figure 4. Purification of light-regulated I_n and II_{pn} NDPK isoforms.
(A) Adsorption chromatography of isoform I_n of NDPK on hydroxyapatite Bio-Gel HTP. (B) Affinity chromatography of isoform I_n of NDPK on GTP-Agarose. (C) Adsorption chromatography of isoform II_{pn} of NDPK on hydroxyapatite Bio-Gel HTP. (D) Affinity chromatography of isoform II_{pn} of NDPK on GTP-Agarose.

polypeptides stained with silver presented in Fig. 5 indicates that both enzyme preparations are homogeneous, and the molecular mass of the purified isoforms is similar or identical, 17–18 kDa. The molecular mass of the native isoforms I_n and II_{pn} obtained after ion exchange chromatography determined by gel filtration on a Sephadex G-150 column is 71 and 63 kDa, respectively, indicating that both isoforms have a homotetrameric structure with a molecular mass of approx. 68 kDa.

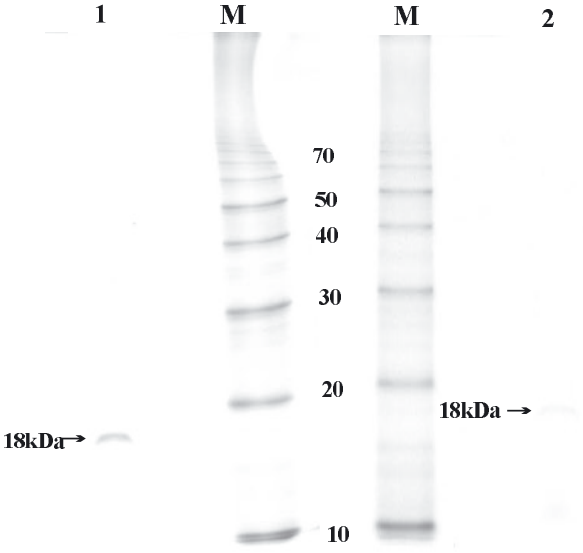

Figure 5. Electrophoresis in 16% polyacrylamide gel with SDS of isoforms I_n and II_{pn} of NDPK.
Lane 1, isoform I_n; lane 2, isoform II_{pn}; M, molecular mass standards. Proteins were visualized by silver staining according to McVeight *et al*. (1988).

In investigations on signal pathways activated by phytochromes, the following proteins have been identified to interact with these photoreceptors: PIFs, HFR/REP1/RSF1, ARR4, ELF, FHY1 (Lorrain *et al*., 2006; Castillon *et al*., 2007), and among them also proteins which undergo phosphorylation as a result of such an interaction: PKS1, Aux/IAA, Cry1, NDPK (Kim *et al*., 2002; Quail, 2002). Our interest in the regulation of NDPK by light through phytochromes follows that this is the only case known to us in which phytochrome A interacts directly with the enzyme protein affecting its catalytic activity. The results of experiments performed on animals, which unequivocally indicate that the role of NDPK in processes occurring in the cell is much broader than it was previously suspected were also important. NDPK, the product of the *awd* gene plays an important role in the development of *D. melanogaster* larvae (Lacombe *et al*., 2000), while human NDP-A kinase encoded by the *NM23-H1* gene was identified for the first time as a protein binding to the sequence silencing the *PDGF* gene (Ma *et al*., 2002). NDP-B kinase, the product of the human *NM23-H2* gene, binds to a site described as PuF/CT in the promoter of the *c-MYC* oncogene (Postel *et al*., 1993; Engel *et al*., 1995; Wagner *et al*., 1997; Hartsough & Steeg, 2000; Iizuka *et al*., 2003). Summing up the re-

sults of relatively numerous investigations, the conclusion may be drawn that some NDPK isoforms are active in regulation of transcription and DNA repair, others participate in the regulation of cell differentiation and yet other NDPK isoforms may play the role of phosphotransferases transferring a phosphate residue to proteins. A number of reports point out to the possibility of interactions of some NDPKs with other proteins, especially various GTP-binding proteins (Kikkawa *et al*., 1990; Bominaar *et al*., 1993; Kimura *et al*., 2000; 2003; Otero, 2000; Baillat *et al*., 2002; Cuello *et al*., 2003; Hippe *et al*., 2003; Narayanan & Ramaswami, 2003).

The first investigations of NDPK from plant material were based on purifying two isoforms from spinach leaves and subsequently on cloning the genes which encoded them (Nomura *et al*., 1991; 1992; Zhang *et al*., 1993). The purified NDPK and NDPKIIs are homohexamers (92–110 kDa) built of polypeptides with a molecular masses of 16 and 18 kDa (Zhang *et al*., 1993). Lübeck & Soll (1995) isolated two isoforms with molecular masses 18.5 and 17.4 kDa from pea chloroplasts and from pea and spinach cytoplasm. NDPKIII with a molecular masses of 16.485 kDa localized in the microsomal fraction was also purified (White *et al*., 1993; Finan *et al*., 1994; Zhang *et al*., 1995). NDPK localized in pea mitochondria is synthesized as a 232-amino-acid polypeptide (26 kDa), from which an 80-amino-acid fragment (9 kDa) is cleaved off during transport (Escobar Galvis *et al*., 1999; Struglics & Hakansson, 1999). In the alga *Dunaliella tertiolecta*, a polypeptide of a similar size and localized in mitochondria is encoded by a nuclear NDPK gene (Anderca *et al*., 2002). Recently, the crystal structure of the mitochondrial NDPK was determined and similarly to enzymes from other organisms it turned out to be a homohexamer (Johansson *et al*., 2004).

The results of investigations performed so far on NDPKs link the functions of various isoforms with plant responses to some stress factors and to light. Using the yeast two-hybrid system with the C-terminal fragment of the *A. thaliana NDPK1* gene, a catalase gene product (AtCAT3) whose expression is regulated by light was identified (Zhong *et al*., 1997; Fukamatsu *et al*., 2003). UV-B light in *A. thaliana* activates the expression of the *NDPKIa* gene encoding a polypeptide containing a lysine residue in positions 113 and 214, which play a key role in interactions with DNA in the human isoforms Nm23-H1 and H2 (Zimmermann *et al*., 1999). In yeast *gcn4* mutants NDPKIa binds to the promoter of the yeast *HIS4* gene coding for one of the enzymes of histidine biosynthesis, activating its expression and reverting the cell phenotype to the wild type (Zimmermann *et al*., 1999). Investigations have also been performed showing that NDPK may be controlled by light as brief irradiation of fragments of etiolated pea and rice seedlings with red light causes phosphorylation of NDPK linked to plasmatic membranes and localized in the cytoplasm (Hamada & Hasunuma, 1994; Hamada *et al*., 1996; 1999; Tanaka *et al*., 1998; Ogura *et al*., 1999).

The participation of phytochromes in red-light-induced regulation of NDPK was unequivocally confirmed by Choi *et al*. (1999). Those authors using the yeast two-hybrid system found that the C-terminal fragment of phytochrome A in *A. thaliana* interacts with recombinant *A. thaliana* NDPK2 and that the physical effect of the interaction between the two proteins is a clear activation of NDPK2. The *A. thaliana ndpk2* mutant growing under continuous far-red light shows a number of phenotype changes that resemble changes characteristic for the *phyA* mutant, whereas in red light *ndpk2* is characterized by a lack of opening and greening of the cotyledons, reactions dependent on phytochrome B (Choi *et al*., 1999).

The results of our experiments indicate that in oat coleoptiles there are several isoforms of NDPK localized mainly in the cytoplasmic fraction as well as in the mitochondrial and etioplastic fractions (altogether about 95% of total activity). About 1% of the total NDPK activity was present in the nuclear fraction. It is notable that in the nuclear fraction obtained from red-light irradiated coleoptiles, NDPK activity increases by about 42% (Table 2). We assume that this is a result of light-regulated nuclear import of the phytochrome A–NDPK complex or that a phytochrome-regulated NDPK isoform localized in nuclei is activated upon phytochrome A translocation into the nuclei (Fankhauser & Chen, 2008). Results shown in Fig. 1 evidence that red, far-red and white light are equally effective in inducing the nuclear accumulation of phytochrome A. On the other hand, only red-light illumination of coleoptiles causes a significant increase of NDPK activity in nuclear fraction (Table 2). These results clearly demonstrated that a phytochome A regulated the NDPK isoform is located in the nuclear fraction.

The total NDPK activity contained in the nuclear fraction is due to two well-separated isoforms of the enzyme (Fig. 2). The isoform eluting from DEAE-Sephacel at 30 mM chloride ion concentration, designated by us as I_n, is clearly activated by phytochrome A in the form of Pfr. Isoform II_n eluting from DEAE-Sephacel only at 150–200 mM chloride ion concentration is not activated by purified phytochrome A (Table 3). From the postnuclear fraction, only the enzyme contained in the activity peak eluting from the column at 30 mM chloride ion concentration is activated by phytochrome A. Purified NDPK preparations susceptible to phytochrome A activation from cell nuclei and the postnuclear

fraction have the same monomeric molecular mass (17–18 kDa). The molecular mass determined by gel filtration on a G-150 column is also similar (71 and 63 kDa), suggesting that the native enzyme is a homotetramer (68 kDa). Taking additionally into consideration the similar conditions of separation on DEAE-Sephacel and hydroxyapatite columns, it can be assumed that the enzyme preparations designated as I_n and II_{pn} are essentially the same NDPK isoform localized partly in the nuclei and partly in the cytoplasm. Such a localization may suggest the possibility of shuttling of this NDPK isoform between the cytoplasm and the nucleus, which similarly as in the case of phytochromes may depend on the light conditions and may be strictly linked to the role of NDPK in phytochrome signaling. On the other hand, the results of experiments presented in Table 3 indicate that the activation of NDPK from nuclei and the postnuclear fraction by phytochrome A in the Pfr form is different. The nuclear enzyme is activated by over 300%, whereas the enzyme from the postnuclear fraction by only 33%. Moreover, the photoconversion of phytochrome A to the Pr form differently affects the NDPK activity. In the case of the enzyme from the nuclear extract, the far-red light did not reverse the effect of red light but increased the enzyme activation by a further 16%, whereas the activity of the enzyme from the postnuclear fraction did not change under these conditions. The observed differences may be explained by the presence of other proteins or other cell components accompanying the NDPKs obtained from the nuclear extract and the postnuclear fraction. The clear NDPK activation in the presence of phytochrome A in the Pfr form should be abolished after irradiation of phytochrome with far-red light (photoconversion of the phytochrome to the Pr form). The lack of such a reversion can be interpreted as a sign of more permanent changes based on, e.g., phosphorylation of the enzyme by phytochrome A and not only on conformational changes caused by a physical interaction between the two proteins. Recent investigations of the interactions of phytochrome A with *A. thaliana* NDPK2 have demonstrated an important role of the phosphorylation and dephosphorylation of a serine residue in position 598 in phytochrome A, one of several serine residues subject to autophosphorylation or phosphorylation by protein kinases (Kim *et al*., 2004; Ryu *et al*., 2005). Phosphoserine-598 dephosphorylation by a specific protein phosphatase PAPP5 allows the interaction of the photoreceptor with NDPK2 (Ryu *et al*., 2005). Conformational changes taking place in the enzyme because of substrate binding also play a role in the binding of NDPK2 by the C-terminal region of phytochrome A (Im *et al*., 2004).

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REFERENCES

Anderca MI, Furuichi T, Pinontoan R, Muto S (2002) Identification of a mitochondrial nucleoside diphosphate kinase from green alga *Dunaliella tertiolecta*. *Plant Cell Physiol* **43**: 1276–1284.

Baillat G, Gaillard S, Castets F, Monneron A (2002) Interactions of phocein with nucleoside-diphosphate kinase, Eps15, and dynamin I. *J Biol Chem* **277**: 18961–18966.

Barthel TK, Walker GC (1999) Inferences concerning the ATPase properties of DnaK and other HSP70s are affected by the ADP kinase activity of copurifying nucleoside-diphosphate kinase. *J Biol Chem* **274**: 36670–36678.

Bölter B, Sharma R, Soll J (2007) Localisation of *Arabidopsis* NDPK2 – revisited. *Planta* **226**: 1059–1065.

Bominaar AA, Molijn AC, Pestel M, Veron M, Van Haastert PJM (1993) Activation of G-proteins by receptor-stimulated nucleoside diphosphate kinase in *Dictyostelium*. *EMBO J* **12**: 2275–2279.

Castillon A, Shen H, Huq E (2007) Phytochrome interacting factors: central players in phytochrome-mediated light signaling networks. *Trends Plant Sci* **12**: 514–521.

Choi G, Yi H, Lee J, Kwon Y-K, Soh MS, Shin B, Luka Z, Hahn T-R, Song P-S (1999) Phytochrome signalling is mediated through nucleoside diphosphate kinase 2. *Nature* **401**: 610–613.

Choi G, Kim J-I, Hong S-W, Shin B, Choi G, Blakeslee JJ, Murphy AS, Seo YW, Kim K, Koh E-J, Song P-S, Lee H (2005) A possible role for NDPK2 in the regulation of auxin-mediated responses for plant growth and development. *Plant Cell Physiol* **46**: 1246–1254.

Cuello F, Schulze RA, Heemeyer F, Meyer HE, Lutz S, Jakobs KH, Niroomand F, Wieland T (2003) Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits. *J Biol Chem* **278**: 7220–7226.

Dorion S, Matton DP, Rivoal J (2006) Characterization of a cytosolic nucleoside diphosphate kinase associated with cell division and growth in potato. *Planta* **224**: 108–124.

Engel M, Veron M, Theisinger B, Lacombe M-L, Seib T, Dooley S, Welter C (1995) A novel serine/threonine-specific protein phosphotransferase activity of Nm23/nucleoside-diphosphate kinase. *Eur J Biochem* **234**: 200–207.

Escobar Galvis ML, Hakansson G, Alexciev K, Knorpp C (1999) Cloning and characterisation of a pea mitochondrial NDPK. *Biochimie* **81**: 1089–1096.

Escobar Galvis ML, Marttila S, Hakansson G, Forsberg J, Knorpp C (2001) Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase with a novel 86-kilodalton protein. *Plant Physiol* **26**: 69–77.

Evans A, Smith H (1976) Localization of phytochrome in etioplasts and its regulation *in vitro* of gibberellin levels. *Proc Natl Acad Sci USA* **73**: 138–142.

Fankhauser C, Chen M (2008) Transposing phytochrome into the nucleus. *Trends Plant Sci* **13**: 596–601.

Finan PM, White IR, Redpath SH, Findlay JBC, Millner PA (1994) Molecular cloning, sequence determination and

heterologous expression of nucleoside diphosphate kinase from *Pisum sativum*. *Plant Mol Biol* **25**: 59–67.

Fukamatsu Y, Yabe N, Hasunuma K (2003) *Arabidopsis* NDK1 is a component of ROS signaling by interacting with three catalases. *Plant Cell Physiol* **44**: 982–989.

Hamada T, Hasunuma K (1994) Phytochrome-mediated light signal transmission to the phosphorylation of proteins in the plasma membrane and the soluble fraction of etiolated pea stem sections. *J Photochem Photobiol B: Biol* **24**: 163–167.

Hamada T, Tanaka N, Noguchi T, Kimura N, Hasunuma K (1996) Phytochrome regulates phosphorylation of a protein with characteristics of a nucleoside diphosphate kinase activity in the crude membrane fraction from stem section of etiolated pea seedlings. *J Photochem Photobiol B Biol* **33**: 143–151.

Hamada T, Hasunuma K, Komatsu S (1999) Phosphorylation of proteins in the stem section of etiolated rice seedling irradiated with red light. *Biol Pharm Bull* **22**: 122–126.

Hammargren J, Salinas T, Maréchal-Drouard L, Knorpp C (2007) The pea mitochondrial nucleoside diphosphate kinase cleaves DNA and RNA. *FEBS Lett* **581**: 3507–3511.

Harlow E, Lane D (1988) *Antibodies. A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Harris N, Taylor JE, Roberts JA (1994) Isolation of a mRNA encoding a nucleoside diphosphate kinase from tomato that is up-regulated by wounding. *Plant Mol Biol* **25**: 739–742.

Hartsough MT, Steeg PS (2000) Nm23/Nucleoside diphosphate kinase in human cancers. *J Bioenerg Biomembr* **32**: 301–308.

Hippe H-J, Lutz S, Cuello F, Knorr K, Vogt A, Jakobs KH, Wieland T, Niroomand F (2003) Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits. *J Biol Chem* **278**: 7227–7233.

Hurkman WJ, Dupont FM, Altenbach SB, Combs A, Chan R, Tanaka CK, Reuveni M, Bernardin JE (1998) BiP, HSP70, NDK and PDI in wheat endosperm. II. Effects of high temperature on protein and mRNA accumulation. *Physiol Plant* **103**: 80–90.

Iizuka N, Mori N, Tamesa T, Tangoku A, Oka M (2003) Telomerase activity and Nm23-H2 protein expression in hepatocellular carcinoma. *Anticancer Res* **23**: 43–48.

Im YJ, Kim J-I, Shen Y, Na Y, Han Y-J, Kim S-H, Song P-S, Eom SH (2004) Structural analysis of *Arabidopsis thaliana* nucleoside diphosphate kinase-2 for phytochrome-mediated light signaling. *J Mol Biol* **343**: 659–670.

Johansson M, Mackenzie-Hose A, Andersson I, Knorpp C (2004) Structure and mutational analysis of plant mitochondrial nucleoside diphosphate kinase. Identification of residues involved in serine phosphorylation and oligomerization. *Plant Physiol* **136**: 3034–3042.

Kikkawa S, Takahashi K, Takahashi K-I, Shimada N, Ui M, Kimura N, Katada T (1990) Conversion of GDP into GTP by nucleoside diphosphate kinase on the GTP-binding proteins. *J Biol Chem* **265**: 21536–21540.

Kim J-I, Kozhukh GV, Song P-S (2002) Phytochrome-mediated signal transduction pathways in plants. *Biochem Biophys Res Commun* **298**: 457–463.

Kim J-I, Shen Y, Han Y-J, Park J-E, Kirchenbauer D, Soh M-S, Nagy F, Schafer E, Song P-S (2004) Phytochrome phosphorylation modulates light signaling by influencing the protein-protein interaction. *Plant Cell* **16**: 2629–2640.

Kimura N, Shimada N, Fukuda M, Ishijima Y, Miyazaki H, Ishii A, Takagi Y, Ishikawa N (2000) Regulation of cellular functions by nucleoside diphosphate kinases in mammals. *J Bioenerg Biomembr* **32**: 309–315.

Kimura N, Shimada N, Ishijima Y, Fukuda M, Takagi Y, Ishikawa N (2003) Nucleoside diphosphate kinases in mammalian signal transduction systems: Recent development and perspective. *J Bioenerg Biomebr* **35**: 41–47.

Lacombe M-L, Milon L, Munier A, Mehus JG, Lambeth DO (2000) The human Nm23/nucleoside diphosphate kinases. *J Bioenerg Biomembr* **32**: 247–258.

Leung S-M, Hightower LE (1997) A 16-kDa protein functions as a new regulatory protein for Hsc70 molecular chaperone and identified a member of the Nm23/nucleoside diphosphate kinase family. *J Biol Chem* **272**: 2607–2614.

Lorrain S, Genoud T, Fankhauser C (2006) Let there be light in the nucleus! *Curr Opin Plant Biol* **9**: 509–514.

Lübeck J, Soll J (1995) Nucleoside diphosphate kinase from pea chloroplasts: purification, cDNA cloning and import into chloroplasts. *Planta* **196**: 668–673.

Ma D, Xing Z, Liu B, Pedigo NG, Zimmer SG, Bai Z, Postel EH, Kaetzel DM (2002) NM23-H1 and NM23-H2 repress transcriptional activities of nuclease-hypersensitive elements in the platelet-derived growth factor-A promoter. *J Biol Chem* **277**: 1560–1567.

McVeigh T, Caffrey P, Owen P (1988) Silver staining of proteins and lipopolysaccharides in SDS-polyacrylamide gels. In *Immunochemical and molecular genetics analysis of bacterial photogens*, Owen P, Foster TJ eds, pp 267–279. Elsevier Science Publishers.

Moisyadi S, Dharmasiri S, Harrington HM, Lukas TJ (1994) Characterization of a low molecular mass autophosphorylating protein in cultured sugarcane cells and its identification as a nucleoside diphosphate kinase. *Plant Physiol* **104**: 1401–1409.

Moon H, Lee B, Choi G, Shin D, Prasad DT, Lee O, Kwak S-S, Kim DH, Nam J, Bahk J, Hong JC, Lee SY, Cho MJ, Lim CO, Yun D-J (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc Natl Acad Sci USA* **100**: 358–363.

Narayanan R, Ramaswami M (2003) Regulation of dynamin by nucleoside diphosphate kinase. *J Bioenerg Biomembr* **35**: 49–55.

Nomura T, Fukui T, Ichikawa A (1991) Purification and characterization of nucleoside diphosphate kinase from spinach leaves. *Biochim Biophys Acta* **1077**: 47–55.

Nomura T, Yatsunami K, Honda A, Sugimoto Y, Fukui T, Zhang J, Yamamoto J, Ichikawa A (1992) The amino acid sequence of nucleoside diphosphate kinase I from spinach leaves, as deduced from the cDNA sequence. *Arch Biochem Biophys* **297**: 42–45.

Ogita Z-I, Markert CL (1979) A miniaturized system for electrophoresis on polyacrylamide gels. *Anal Biochem* **99**: 233–241.

Ogura T, Tanaka N, Yabe N, Komatsu S, Hasunuma K (1999) Characterization of protein complexes containing nucleoside diphosphate kinase with characteristics of light signal transduction through phytochrome in etiolated pea seedlings. *Photochem Photobiol* **69**: 397–403.

Otero AS (2000) NM23/Nucleoside diphosphate kinase and signal transduction. *J Bioenerg Biomembr* **32**: 269–275.

Parks RE Jr, Agarwal RP (1973) Nucleoside diphosphokinases. In *The Enzymes*, Boyer PD ed, vol 8, pp 370–344. Academic Press, New York.

Postel EH, Berberich SJ, Flint SJ, Ferrone CA (1993) Human *c-myc* transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* **261**: 478–480.

Quail PH (2002) Phytochrome photosensory signaling networks. *Nat Rev* **3**: 85–93.

Ryu JS, Kim J-I, Kunkel T, Kim B-C, Cho DS, Hong SH, Kim S-H, Fernandez AP, Kim Y, Alonso JM, Ecker JR, Nagy F, Lim PO, Song P-S, Schafer E, Nam HG (2005) Phytochrome-specific type 5 phosphatase controls light signal flux by enhancing phytochrome stability and affinity for a signal transducer. *Cell* **120**: 395–406.

Struglics A, Hakansson G (1999) Purification of a serine and histidine phosphorylated mitochondrial nucleoside diphosphate kinase from *Pisum sativum Eur J Biochem* **262**: 765–773.

Tanaka N, Ogura T, Noguchi T, Hirano H, Yabe N, Hasunuma K (1998) Phytochrome-mediated light signal are transduced to nucleoside diphosphate kinase in *Pisum sativum* L. cv. Alaska. *J Photochem Photobiol B: Biol* **45**: 113–121.

Vierstra RD, Quail PH (1983) Purification and initial characterization of 124-kilodalton phytochrome from *Avena*. *Biochem* **22**: 2498–2505.

Wagner PD, Steeg PS, Vu N-D (1997) Two-component kinase like activity of nm23 correlates with its motility-suppressing activity. *Proc Natl Acad Sci USA* **94**: 9000–9005.

White IR, Finan PM, Millner PA (1993) Nucleoside diphosphate kinase associated with *Pisum sativum* microsomal membranes: apparent binding of GTP S at nM concentration. *J Plant Physiol* **142**: 191–196.

Yang KA, Moon H, Kim G, Lim CJ, Hong JC, Lim CO, Yun D-J (2003) NDP kinase 2 regulates expression of antioxidant genes in *Arabidopsis*. *Proc Japan Acad* **79**: 86–91.

Zhang J, Nomura T, Yatsunami K, Honda A, Sugimoto Y, Moriwaki T, Yamamoto J, Ohta M, Fukui T, Ichikawa A (1993) Nucleotide sequence of the cDNA encoding nucleoside diphosphate kinase II from spinach leaves. *Biochim Biophys Acta* **1171**: 304–306.

Zhang J, Fukui T, Ichikawa A (1995) A third nucleoside diphosphate kinase from spinach leaves: purification, characterization and amino-acid sequence. *Biochim Biophys Acta* **1248**: 19–26.

Zhong HH, Resnick AS, Straume M, Mcclung CR (1997) Effects of synergistic signaling by phytochrome A and cryptochrome 1 on circadian clock-regulated catalase expression *Plant Cell* **9**: 947–955.

Zimmermann S, Baumann A, Jaekel K, Marbach I, Engelberg D, Frohnmeyer H (1999) UV-responsive genes of *Arabidopsis* revealed by similarity to the Gcn4-mediated UV response in yeast. *J Biol Chem* **274**: 17017–17024.