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ORIGINAL ARTICLE

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Characterization of a cytosolic nucleoside diphosphate kinase associated with cell division and growth in potato

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Abstract A cDNA encoding *Solanum chacoense* cytosolic NDPK (NDPK1, EC 2.7.4.6) was isolated. The open reading frame encoded a 148 amino acid protein that shares homology with other cytosolic NDPKs including a conserved N-terminal domain. *S. chacoense* NDPK1 was expressed in *Escherichia coli* as a 6×His-tagged protein and purified by affinity chromatography. The recombinant protein exhibited a pattern of abortive complex formation suggesting that the enzyme is strongly regulated by the NTP/NDP ratio. A polyclonal antibody generated against recombinant NDPK1 was specific for the cytosolic isoform in *Solanum tuberosum* as shown from immunoprecipitation experiments and immunoblot analysis of chloroplasts and mitochondria preparations. NDPK activity and NDPK1 protein were found at different levels in various vegetative and reproductive tissues. DEAE fractogel analyses of NDPK activity in root tips, leaves, tubers and cell cultures suggest that NDPK1 constitutes the bulk of extractable NDPK activity in all these organs. NDPK activity and NDPK1 protein levels raised during the exponential growth phase of potato cell cultures whereas no rise in activity or NDPK1 protein was observed when sucrose concentration in the culture was manipulated to limit growth. Activity measurements, immunoblot analysis as well as immunolocalization experiments performed on potato root tips and shoot apical buds demonstrated that NDPK1 was predominantly localized in the meristematic zones and provascular tissues of the apical regions. These data suggest that NDPK1 plays a specific role in the supply of UTP during early growth of plant meristematic and provascular tissues.

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Abbreviations IPTG: Isopropyl β-D-thiogalactoside · BCIP: 5-bromo,4-chloro,3-indolylphosphate · GAPDH: Glyceraldehyde 3-phosphate dehydrogenase · MES: 2-[*N*-morpholino]ethanesulphonic acid · NDPK: Nucleoside diphosphate kinase · Ni-NTA: Ni-nitrilotriacetic acid · PMSF: Phenylmethylsulphonylfluoride · PEPC: Phospho*enol*pyruvate carboxylase · PVPP: Insoluble polyvinylpolypyrrolidone · TEV: Tobacco etch virus

Introduction

Nucleoside diphosphate kinase (NDPK, EC 2.7.4.6) catalyses the transfer of the γ-phosphate of a donor nucleoside triphosphate (NTP) to an acceptor nucleoside diphosphate (NDP). While ATP is generally considered as the preferred donor in vivo, NDPK also transfers the γ-phosphate from other donor NTPs (Lascu and Gonin 2000). This enzyme is widely distributed and is believed to play a role in the general homeostasis of cellular nucleoside triphosphate pools. In prokaryotes, NDPK has been shown to perform a metabolic housekeeping function because of its involvement in the generation of NTPs from ATP (Bernard et al. 2000). This basic function may have been maintained in animals (Lambeth et al. 1997) and plants (Dancer et al. 1990a; Roberts et al. 1997).

In addition to this metabolic role, a number of other functions have been assigned to NDPK which has been extensively characterized in prokaryotes, lower eukaryotes and animal models. These functions are mediated by the ability of various NDPKs to act as a transcriptional regulator (Agou et al. 2000), as a protein phosphotransferase (Freije et al. 1997) or to interact with

other protein partners (Lombardi and Mileo 2003). For example, the products of *NM23* genes in mammalian cells have been identified as NDPK isoforms that are involved in processes affecting cell proliferation through transcriptional regulation and protein–protein interactions (Roymans et al. 2002). Another example of alternate function for NDPK comes from *Drosophila* where mutations in a homolog of *NM23-H1*, the *AWD* gene, impair normal larval development and even cause lethality after the larval stage in the case of null mutations (Timmons and Shearn 2000). The implication of *AWD* in development has been linked to the protein phosphotransferase activity detected in the NDPK encoded by the gene (Inoue et al. 1996).

Our understanding of plant NDPK function(s) is still very patchy. However, it is established that different types of NDPK (isoforms) are present in the cytosolic, plastidic and mitochondrial compartments. Type I NDPK (NDPK1) lacks an identifiable targeting sequence and is therefore probably localized in the cytosol while types II and III (respectively NDPK2 and NDPK3) have N-terminal extensions that suggest organellar targeting (Sweetlove et al. 2001). NDPK1 has been characterized and cloned from several sources but information on the corresponding proteins is still very scarce (Nomura et al. 1991, 1992; Finan et al. 1994; Yano et al. 1995; Zhang et al. 1995). An exception to this is the spinach leaf enzyme, which has been purified and partially characterized (Nomura et al. 1991). In rice, NDPK1 appears to have a positive effect on coleoptile extension (Pan et al. 2000).

In contrast, the characterization of type II and III NDPKs is more advanced. NDPKs have been purified and characterized from chloroplasts (Lubeck and Soll 1995; Yang and Lamppa 1996; Spetea et al. 2004) and shown to be targeted to the stroma (Lubeck and Soll 1995) and the thylakoid lumen (Spetea et al. 2004). The lumen isoform generates GTP for OEC33, a GTP-binding subunit of PSII (Spetea et al. 2004). NDPK activity and protein have also been identified in mitochondria (Escobar Galvis et al. 1999) and shown to be localized in the mitochondrial intermembrane space (Sweetlove et al. 2001). Mitochondrial NDPK oligomerization state has recently been characterized by mutational analysis and crystallography (Johansson et al. 2004). This isoform is probably involved in energy metabolism and stress response since it has been shown to interact with the mitochondrial adenine nucleotide translocator (Knorpp et al. 2003) and a heat-inducible 86-kDa protein (Escobar Galvis et al. 2001). Several lines of evidence also support the involvement of organellar NDPK in signal transduction. In *Arabidopsis thaliana*, a NDPK2 isoform has been identified as a component of the UV response (Zimmermann et al. 1999). This protein interacts with the C-terminal domain of phytochrome A (Shen et al. 2005) and has been shown to belong to the phytochrome-mediated light signalling pathway (Tanaka et al. 1998; Choi et al. 1999). It is also involved in the mitogen-activated protein kinase H_2O_2 signalling pathway (Moon et al. 2003). There is also evidence that a yet uncharacterized, membrane-localized, NDPK becomes phosphorylated following ethylene treatment, suggesting that this protein could be involved in the ethylene signal transduction pathway (Novikova et al. 2003).

From the above data, it appears that NDPK2 has functions that go far beyond the maintenance of NTP pools. Before exploring if other NDPK isoforms could also fulfil diverse roles in plant biology, we need to characterize them and understand where they are expressed in the plant. This is particularly true of type I NDPKs, which are by far the least characterized of all the plant NDPK isoforms. We therefore undertook this study to gain information on the properties and expression of the cytosolic NDPK isoform, with the long-term goal of understanding the function of this isoform in plant biology and metabolism. We present evidence that, in potato, NDPK1 is found in all plant tissues and always accounts for the majority of extractable NDPK activity. High levels of NDPK1 expression are associated with root and shoot meristematic regions as well as provascular tissues. These findings are discussed in relation to the possible metabolic functions of this isoform.

Materials and methods

Chemicals, biological materials and plant culture conditions

Except when mentioned otherwise, buffers, chemicals and reagents were of analytical grade from Sigma Chemical Co. (St Louis, MO) or Fisher Scientific (Nepean, ON, Canada). Percoll was from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada) and Fractogel EMD DEAE-650(S) was from VWR (Mississauga, ON, Canada). Hen anti-PsbA, goat anti-rabbit IgG (Fc)-alkaline phosphatase conjugate and rabbit anti-chicken IgY (H + L) immune serum conjugated to horseradish peroxidase sera were from Agrisera (Vännäs, Sweden), Promega (Nepean, ON, Canada) and Amersham Pharmacia Biotech (Baie d'Urfé, Qc, Canada), respectively. Seed potatoes (cv Russet Burbank) were a generous gift from Propur Inc. (Saint-Ambroise, QC, Canada). Potato plants were grown as described (Dorion et al. 2005). Potato cell cultures were maintained and harvested by centrifugation as described (Dorion and Rivoal 2003). Plant samples were collected, weighed, frozen in liquid nitrogen and stored at −80°C until used. In the case of root tips, plant material was carefully washed in deionized water followed by blotting before freezing. For immunolocalization studies, fresh material was used.

cDNA cloning and sequencing

A full-length NDPK1 was obtained from a *S. chacoense* (a wild relative of potato) EST collection generated from

weakly expressed mRNAs in pistil tissues (Lantin et al. 1999). A single cDNA encoding for a full-length NDPK1 was found. The cDNA was completely sequenced on both strands using the Big Dye Terminator 2.0 sequencing kit (Perkin Elmer, Montréal, QC, Canada) and an ABI 310 automated sequencer. The sequence data have been deposited with the NCBI/GenBank data libraries under accession number DQ157699.

Construct for recombinant NDPK1 expression in *Escherichia coli* and purification of the recombinant protein

The NDPK1 cDNA in pBKCMV was amplified using the forward primer NDPKF1 5′-ATGGAGCAGACT TTCATCATGATC-3′ and the reverse primer NDPKR1 5′-CGAATTGGGTACACTTACCTGGTA-3′. The 5′ end of the resulting PCR product coincided with NDPK1 initiation codon and the 3′ end contained vector sequences. This 776 bp fragment was digested with *Xba*I and the resulting 710 bp restriction fragment carrying the full coding sequence was cloned into *Ehe*I/*Xba*I digested pProEx HTb (Invitrogen Canada Inc, Burlington, ON, Canada). The ligated plasmid was used to transform competent *E. coli* cells (DH5α strain). The construction was verified by full sequencing. The expression plasmid carried the entire coding sequence of *S. chacoense* NDPK1 in frame with a 24 amino acids extension located at the N-terminus encoding for a (6×His) tag and the tobacco etch virus (TEV) protease cleavage site. The deduced amino acid sequence of the recombinant protein was 172 amino acids in length and encoded a polypeptide with a M_r of 19,300.31. *E. coli* carrying the construct encoding recombinant NDPK1 was grown in Luria-Bertani broth medium (500 ml total volume) at 37°C to an A_{600} of 0.5. IPTG was then added to the culture at a final concentration of 0.6 mM and bacteria were grown for an additional 4 h. Cells were harvested by centrifugation (10 min, 5,000×*g*) and the pellets frozen at −80°C until used. For the purification of recombinant NDPK1, the steps were performed at room temperature under native conditions on a 1 ml column of Ni-NTA according to the manufacturer's instructions (Invitrogen Canada Inc, Burlington, ON, Canada). The purified fractions were pooled, dialyzed against a buffer containing 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DTT and used to prepare the anti-NDPK1 immune serum. (6×His) NDPK1 was also used for determination of kinetic parameters. In this case, the dialyzed enzyme preparation was stored at −20°C in 50% (v/v) glycerol until used.

Production of anti-NDPK1 immune serum

Affinity-purified (6×His)-epitope tagged NDPK1 was incubated with TEV protease (Invitrogen Canada Inc, Burlington, ON, Canada) for 48 h at room temperature. This only resulted in a partial (20–30%) digestion of recombinant NDPK1. Following digestion, the polypeptide mixture was separated by SDS/PAGE. After electrophoresis, polypeptides were stained with Coomassie blue for 5 min followed by 15 min destaining. The band corresponding to de-tagged NDPK1 (M_r = 16,300) was excised and electroeluted from the gel using a Bio-Rad electroeluter according to the directions of the manufacturer. The electroeluted protein solution (approximately 250 µg protein in 1 ml) was used to raise a polyclonal immune serum in a 2 kg New-Zealand rabbit. After collection of the pre-immune serum, 100 µg NDPK1 protein (emulsified in complete Freund's adjuvant) was injected subcutaneously into the back of the rabbit. Booster injections were administered on days 14 and 21 with 75 µg recombinant NDPK freshly emulsified in incomplete Freund's adjuvant. Fourteen days after the final injection, blood was collected by cardiac puncture. Anti-NDPK1 IgGs were affinity purified as described (Dorion et al. 2005) against 20 µg of de-tagged NDPK1. The serum was collected after centrifugation at 1,500×*g*, frozen in aliquots in liquid N_2 and kept at −80°C until used.

Enzyme extraction from plant tissues, protein assays, determination of NDPK activity and kinetic parameters

All steps were carried out at 4°C. All plant tissues except cell cultures were ground with sand with a pestle and mortar using a ratio of 2:1 (ml extraction buffer/g fresh weight) in a buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 10 mM EDTA, 1% (v/v) triton, 0.5% (w/v) Na deoxycholate, 1 mM Na_3V0_4, 5 mM DTT, 5% (w/v) insoluble PVPP and 1 mM PMSF. Potato cell cultures were homogenized in the above buffer using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON, Canada) equipped with a PTA 20S probe (3×10 s bursts at maximum power) (Dorion and Rivoal 2003). The homogenate was centrifuged for 15 min at 15,000×*g*. The resulting supernatant was desalted on PD10 columns (Pharmacia) against a buffer containing 50 mM Tris–HCl pH 7.5, 10% (v/v) glycerol, 2.5 mM $MgCl_2$ and 1 mM DTT. Desalted extracts were used immediately for protein and enzyme activity measurement. In cases where proteins were being analyzed by immunoblot, an aliquot of the extract was immediately heat-denatured in SDS sample buffer and kept frozen at −20°C until analysis. NDPK activity measurements in desalted plant extracts were performed using a previously described HPLC assay, which allows accurate determination of NDPK activity without interference from enzymes degrading NTPs (Dorion and Rivoal 2003). This assay was also used to determine the affinity of NDPK for GTP. In that case, GTP was used instead of CTP. A coupled enzyme spectrophotometric assay was also used with partially purified and pure NDPK (Dorion and Rivoal 2003).

One unit of enzyme activity (U) corresponds to the appearance of the reaction product at the rate of 1 μmol/min. Proteins were quantified on a VERSAmax microplate reader (Molecular Devices, Sunnyvale CA) by the method of Bradford (Bradford 1976) using BSA as standard and the protein assay kit from Bio-Rad (Mississauga, ON, Canada). For the analysis of recombinant NDPK1 kinetic parameters, HPLC and spectrophotometric assays were used with the following modifications. For optimum pH determination, the reactions were carried out in a three buffer system (Acetic acid/MES/Tris) in order to keep a constant ionic strength across the pH range (Ellis and Morrison 1982). For the determination of apparent K_m values, the concentration of one substrate varied while the other was kept at saturation. Analysis of the kinetic parameters was done using a non-linear regression analysis software (SigmaPlot 8.02, SPSS Inc. Chicago, IL). Experimental data were fitted to a Michaelis–Menten equation and results are the mean (SE for at least three independent determinations.

Chloroplasts isolation from potato leaves

All procedures were carried out at 4°C. The isolation method (Mills and Joy 1980) was optimized for the recovery of pure and intact potato leaf chloroplasts (Dorion et al. 2005). Organelle purity was evaluated by assaying phospho*enol*pyruvate carboxylase (EC 4.1.1.31) as cytosolic marker (Rivoal et al. 1996) and NADP-dependent GAPDH (NADP–GAPDH, EC 1.2.1.13) as chloroplastic marker (Rivoal et al. 1989). No PEPC activity was detected in Percoll-purified chloroplasts. Chloroplasts intactness (70–75%) was assessed using NADP–GAPDH activity before and after breaking the chloroplasts in homogenization medium without sorbitol.

Preparation of mitochondria from potato tubers

All procedures were done at 4°C. The method for preparation of washed mitochondria from potato tuber was adapted from Berry et al. (1991), De Leonardis et al. (1995) and Rothbauer et al. (2001). Whole potato tubers (100 g) were cut in 1 cm^3, then transferred to 300 ml of extraction buffer (0.1 M K_2HPO_4, pH 7.4, 0.3 M sucrose, 7 mM L-cysteine, 0.25% (w/v) de-fatted BSA) and disrupted in a blender at high speed for 30 s. The homogenate was then filtered through 5–10 layers of cheesecloth and centrifuged for 5 min at 600×*g*. The pellet which contained nuclei, chloroplasts, intact cells and membranes was discarded and the supernatant was centrifuged for 15 min at 2,000×*g*. The resulting supernatant was centrifuged for 20 min at 8,000×*g*. To prepare the washed mitochondria, the 8,000×*g* pellet was resuspended in 8 ml of wash medium (10 mM K_2HPO_4, pH 7.2, 0.3 M sucrose, 0.1% (w/v) de-fatted BSA, 1 mM EDTA) and centrifuged for 10 min at 250×*g*. The pellet was discarded and the supernatant was centrifuged again for 10 min at 250×*g*. The resulting supernatant was centrifuged for 15 min at 6,000×*g*. The pellet (washed mitochondria) was resuspended in 1 ml of wash medium. Organelle purity was evaluated by assaying PEPC (EC 4.1.1.31) as cytosolic marker (Rivoal et al. 1996) and fumarase (EC 4.2.1.2) as mitochondrial marker (Behal and Oliver 1997). No PEPC activity was detected in washed mitochondria fraction.

NDPK1 immunoprecipitation

All steps were done at 4°C. NDPK1 was subjected to immunoprecipitation from 1.5 g of potato cells sampled from the logarithmic growth phase (7 days). Crude cell extracts were prepared in immunoprecipitation buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 10 mM EDTA, 1% (v/v) triton, 0.5% (w/v) Na deoxycholate, 1 mM Na_3V0_4, 5 mM DTT and 1 mM PMSF). Clarified extracts were obtained by homogenizing cells with a polytron as described above. The homogenate was centrifuged for 15 min at 15,000×*g*. Supernatants were incubated for 90 min with 100 μl of immune serum or pre-immune serum. Antigen–antibody complexes were incubated for 30 min with 75 μl protein A-Sepharose beads (220 μg protein A) previously equilibrated in immunoprecipitation buffer and centrifuged for 1 min at 15,000×*g*. Pellets were washed five times with 500 μl immunoprecipitation buffer. Immunoprecipitates were solubilized by boiling 2.5 min in SDS sample buffer without DTT and immediately analyzed by SDS/PAGE. For N-terminal sequencing, immunoprecipitates were processed as described below.

SDS/PAGE and immunoblot analysis

SDS/PAGE analysis was performed according to (Laemmli 1970) on 15% acrylamide gels. Blotting was done as described by (Rivoal et al. 2001). Blots were incubated with anti-NDPK1 affinity purified IgGs (1/50 dilution), affinity-purified anti-cytosolic castor oil seed endosperm aldolase IgG (1/100 dilution), anti-cytosolic triose phosphate isomerase antibody (1/500 dilution) (Dorion et al. 2005) or anti-cytochrome c mouse mAb clone 7H82C2 (1/2,000 dilution, Pharmingen, Oakville, ON, Canada) for 1 h at room temperature. Polypeptides were detected using the following secondary antibodies conjugated to alkaline phosphatase: rabbit anti-mouse IgG (1/2,500 dilution) (ICN, Aurora OH) and goat anti-rabbit IgG (1/10,000 dilution) (Promega, Missisauga, ON, Canada). The reaction was visualized using BCIP and nitroblue tetrazolium and was routinely allowed to develop for 2–5 min at room temperature. Immunoblots incubated with the pre-immune serum gave negative results. For PAGE and immunodetection analysis of the photosystem II protein PsbA (chloroplast marker), a

previously described procedure was used (Dorion et al. 2005).

N-terminal sequencing

Immunoprecipitates were separated by SDS-PAGE as described above except that 0.1 mM Na thioglycolate was added to the upper buffer chamber. Following electrophoresis, the gel was soaked for 10 min in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulphonic acid, pH 11, 10% (v/v) methanol), then transferred onto a Bio-Rad Sequiblot membrane for 60 min at 70 V. The membrane was washed in milliQ H_2O and stained for 5 min with 0.1% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol. The membrane was then de-stained for a total of 7 min with 5 changes of de-staining solution [methanol/acetic acid/water (50/10/40) (v/v/v)]. After rinsing (5×2 min) with 20 ml MilliQ H_2O, and air-drying, the NDPK1 band on the membrane (approximately 3 µg protein) was subjected to automated Edman degradation.

Analytical anion exchange chromatography

NDPK activity was analysed by Fractogel EMD DEAE-650 (S) ion-exchange chromatography. NDPK was extracted and clarified by centrifugation as described above. The clarified extract was desalted in buffer A [Tris-Cl 20 mM, pH 8, EDTA 1 mM, $MgCl_2$ 1 mM, glycerol 10% (v/v), DTT 1 mM] using PD-10 columns. Desalted extract (2 mg protein) was loaded at 0.5 ml/min onto a column (1 cm × 8 cm) of Fractogel EMD DEAE-650 (S) pre-equilibrated in buffer A. The column was connected to a Pharmacia FPLC system and washed with 18 ml buffer A. Approximately 10% of starting NDPK activity was recovered in the flow-through. Bound activity was eluted with a 72-ml linear gradient of (0–500) mM KCl in buffer A followed by a step gradient to 1 M KCl in buffer A. Fractions of 1 ml were collected and assayed. Activity recovery after separation was high (>90% of starting activity). NDPK activity was resolved in two discrete peaks eluting between 100 and 200 mM KCl. Both peaks typically eluted over 1–2 column volumes. These results were reproduced three times with different batches of material, and similar results were obtained. Representative elution profiles are shown. An aliquot of fractions containing NDPK activity was further analysed by SDS/PAGE and immunoblot using anti-NDPK1 affinity purified IgGs. Representative blots are shown.

Immunocytochemical localization of NDPK1

Fixation and embedding of plant tissues was largely done according to St-Pierre et al. (1999). Briefly, root tips or germinating buds from 1- to 2-week-old potato plants were infiltrated in vacuo in FAA (50% ethanol, 5% acetic acid and 5% formaldehyde) for 20 min and transferred to fresh FAA at 4°C for 16 h with shaking. Tissues were dehydrated using a series of ethanol and *ter*-butanol ending by an overnight incubation at 28°C in pure *ter*-butanol. The day after, samples were incubated at 62°C first in Paraplast Plus:*ter*-butanol (1:1) for 3 h, then in pure Paraplast Plus for 5 h and finally transferred to fresh Paraplast Plus overnight. Paraplast embedded samples were sectioned to a thickness of 10 µm using a Leica microtome. Sections were spread on slides pre-treated with 2% (v/v) 3-aminopropyltriethoxysilane in acetone, dried for 24 h at 37°C and stored until used. Two 15-min incubations in xylene were used to remove Paraplast from the samples, and an ethanol series up to water was used to re-hydrate the sections. For immunolocalization of NDPK1, sections were re-hydrated, as described above, washed in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 10 min, and treated with blocking solution (3% BSA in TBST) for 16 h at 4°C. After washing with TBST, sections were incubated with primary crude antisera at a 1:25 dilution in blocking solution for 3 h at room temperature. After four 15-min washes in TBST, the sections were incubated with goat anti-rabbit IgG (Fc)-alkaline phosphatase conjugate at a 1:1,000 dilution in blocking solution. After 2 h incubation at room temperature, sections were washed twice for 15 min with TBST and twice for 15 min with carbonate buffer (100 mM $NaHCO_3$, pH 9.8, and 1 mM $MgCl_2$). The slides were incubated with 150 µg/ml BCIP and 300 µg/ml nitro blue tetrazolium chloride in carbonate buffer for 10 min at room temperature. After development, slides were washed in water followed by an ethanol series up to xylene and covered with a cover slip after adding Permount to preserve the tissues.

Results

Cloning and sequence analysis of a full-length cDNA encoding for NDPK1

A 753 bp cDNA encoding for *Solanum chacoense* NDPK1 was isolated from a *S. chacoense* EST collection. cDNA sequencing and conceptual translation revealed a 148 amino acids open reading frame that shared homology with other NDPKs (Fig. 1). The highest homology was observed with type I NDPKs (e.g. Swiss-Prot accession numbers Q9M7P6, Q02254 and P39207). These proteins lack the N-terminal extension found in type II and type III NDPKs. Dicotyledon NDPK1s have a consensus N-terminal sequence (MEQTFI) found in the *S. chacoense* NDPK. All NDPKs share a catalytic His residue which autophosphorylates as part of the catalytic mechanism (Lascu and Gonin 2000). This residue is found at position 115 in *S. chacoense* NDPK1 (Fig. 1). A phylogenic analysis of *S. chacoense* NDPK1 and other plant NDPK sequences was performed

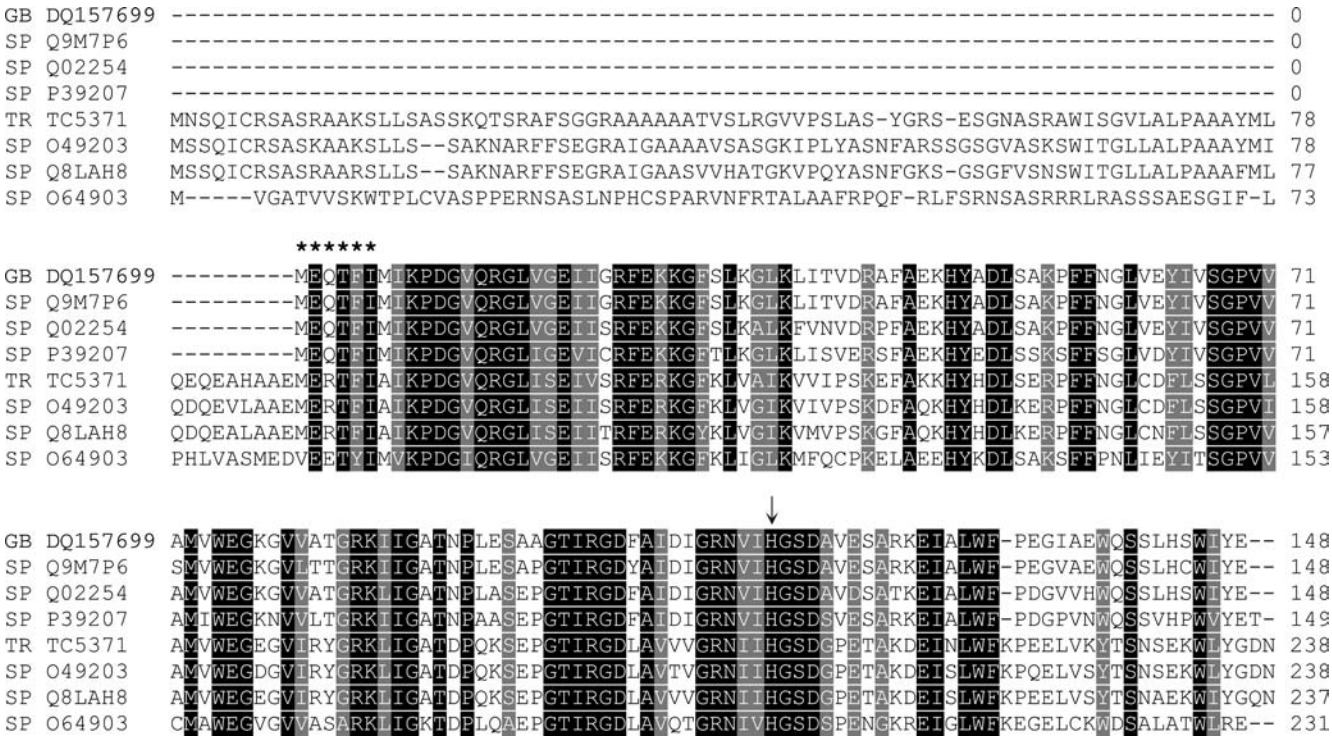


Fig. 1 Sequence analysis of *S. chacoense* NDPK1. Derived amino acid sequence of the *S. chacoense* NDPK1 cDNA was compared to those of other plant NDPKs using CLUSTAL W (1.74). Gaps were introduced to achieve maximum homology between sequences. Conserved and similar amino acid residues are shaded in *dark* and *light grey*, respectively. The numbering on the *right* refers to each sequence. The consensus N-terminal sequence found in dicotyledon NDPK1s (MEQTFI) is indicated by *asterisks* and the conserved catalytic histidine residue is marked with a *down arrow*. The accession numbers from GenBank (*GB*), Swiss-Prot (*SP*) and TIGR (*TR*) correspond to the following sequences DQ157699: *S. chacoense* NDPK1 (this study), Q9M7P6: *Capsicum annuum* root NDPK; Q02254: *Spinacia oleracea* leaf NPDK1 (Nomura et al. 1992); P39207: *Arabidopsis thaliana* NDPK1; TC5371: NDPK found in the intermembrane space of *Solanum tuberosum* mitochondria (Sweetlove et al. 2001); O49203: NDPK found in the thylakoid lumen (Spetea et al. 2004); Q8LAH8: *Arabidopsis thaliana* NDPK possibly localized in mitochondria (Kruft et al. 2001); O64903: *Arabidopsis thaliana* NDPK involved in phytochrome signalling (Shen et al. 2005) and UV response (Zimmermann et al. 1999)

(Fig. 2). The sequences form three groups that correspond to the three known types of NDPKs. Experimental evidence indicates that cluster II (NDPK2s) contains isoforms found in the chloroplast [e.g. accession number P47923 (Lubeck and Soll 1995)] and that cluster III (NDPK3s) contains isoforms occurring in

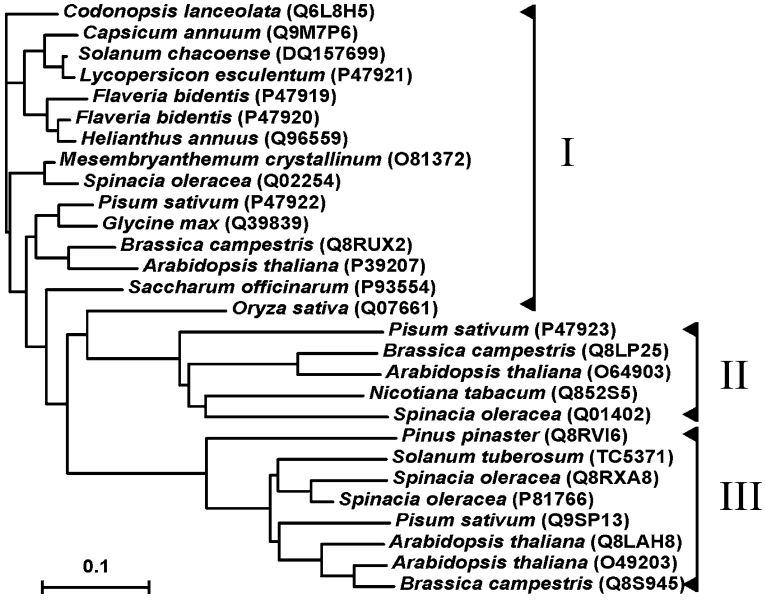


Fig. 2 Phylogenetic tree of plant NDPK sequences. Sequences were aligned as in Fig. 1 and the TreeView 1.6.6 software was used to visualize the tree. The *scale bar* at the bottom of the figure represents 0.1 amino acid substitutions per site. Accession number of each sequence is given in *parenthesis* after the name of the species. *Roman numbers* on the *right* refer to isoform type and were assigned based on database annotations or published experimental evidence

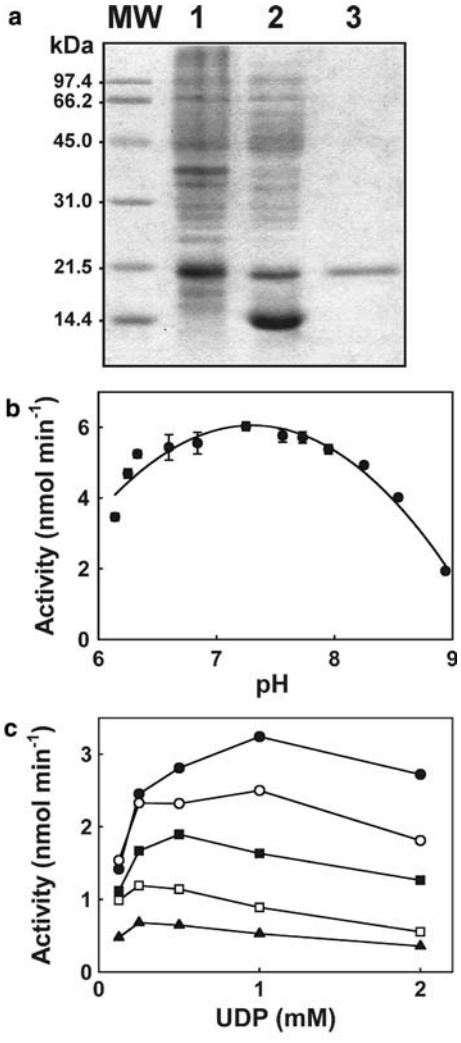
mitochondria [e.g. accession number Q9SP13 (Escobar Galvis et al. 1999)] and the thylakoid lumen [e.g. accession number O49203 (Spetea et al. 2004)]. The *S. chacoense* sequence clearly groups with isoforms identified as cytosolic (Type I) NDPKs (Nomura et al. 1992; Escobar Galvis et al. 1999) and was therefore identified as NDPK1.

Kinetics parameters of recombinant (6×His)-tagged NDPK1

S. chacoense NDPK1 was cloned in the bacterial expression vector pProEX HTb and expressed in *E. coli* DH5α. The recombinant protein was purified under native conditions using immobilized Ni^{2+} affinity resin. SDS/PAGE analysis of the affinity column eluate demonstrated electrophoretic homogeneity of the enzyme preparation (Fig. 3a, lane 3). (6×His)-NDPK1 had an apparent molecular weight of 19.3 kDa, as predicted from the primary sequence. The kinetic parameters of the enzyme were determined using spectrophotometric and HPLC assays. (6×His)-NDPK1 was assayed at various pH values between 6 and 9. The enzyme displayed a broad pH optimum in the range 6.5–8 (Fig. 3b). Several reports (Parks and Agarwal 1973; Dorion and Rivoal 2003) indicate that NDPKs may form abortive complexes when binding their substrates depending on the relative concentrations of NDP and NTP. To study this effect, we assayed recombinant (6×His)-NDPK1 activity at several fixed concentrations of CTP while varying UDP concentration in the reaction mixture. The results (Fig. 3c) clearly show a pattern of abortive complex formation (Parks and Agarwal 1973), i.e. inhibition of the NDPK reaction at high UDP concentrations. Interestingly, *S. chacoense* NDPK1 activity appears more sensitive to high NTP/NDP ratios than yeast NDPK when assayed under the same conditions (Dorion and Rivoal 2003). The affinity of (6×His)-NDPK1 for substrates used in the spectrophotometric and HPLC assays was also determined (Fig. 3d). Among the donors, calculated K_ms of NDPK for its substrates were GTP < ATP < CTP in order of increasing value. For acceptors, apparent K_m for UDP was lower than for TDP.

Specificity of anti-NDPK1 immune serum

A polyclonal immune serum was raised in rabbit by injecting the recombinant protein after removing the (6×His)-tag. To test the specificity of this anti-NDPK1 serum, crude potato cell extracts were subjected to immunoprecipitation with the immune serum or the corresponding pre-immune serum (Fig. 4a). Immunoprecipitated proteins were analysed by SDS-PAGE followed by Coomassie staining (Fig. 4a, upper panel). The majors bands common to the two sera, most likely correspond to the IgGs heavy and light chains. A



d

Substrate	K_m (μM)
ATP	400 ± 20
CTP	1030 ± 10
GTP	75 ± 2
TDP	1170 ± 60
UDP	210 ± 50

Fig. 3 Characterization of recombinant (6×His)-NDPK1. **a** SDS/PAGE analysis of recombinant NDPK1 purification. *Lanes* are: *MW* molecular weight standards, *1* IPTG-induced *E. coli* crude extract, *2* clarified supernatant, *3* Ni-NTA purified (6×His)-NDPK1. Protein bands were visualized by Coomassie staining. **b** Effect of pH on recombinant NDPK1 activity. Activity was measured using a three buffer mixture (25 mM acetic acid, 25 mM MES, 50 mM Tris) and pH adjusted with NaOH or HCl to the desired value. **c** Effect of varying the CTP/UDP ratio on NDPK1 activity: 3 mM CTP (*filled circle*), 2 mM CTP (*open circle*), 1 mM CTP (*filled square*), 0.5 mM CTP (*open square*) and 0.25 mM CTP (*filled triangle*). **d** Summary of K_m values of recombinant NDPK1 for substrates used in the HPLC (CTP, GTP and UDP) and spectrophotometric (ATP and TDP) assays

polypeptide was specifically immunoprecipitated by the immune serum. This polypeptide was detected by immunoblot analysis of the immunoprecipitate by the

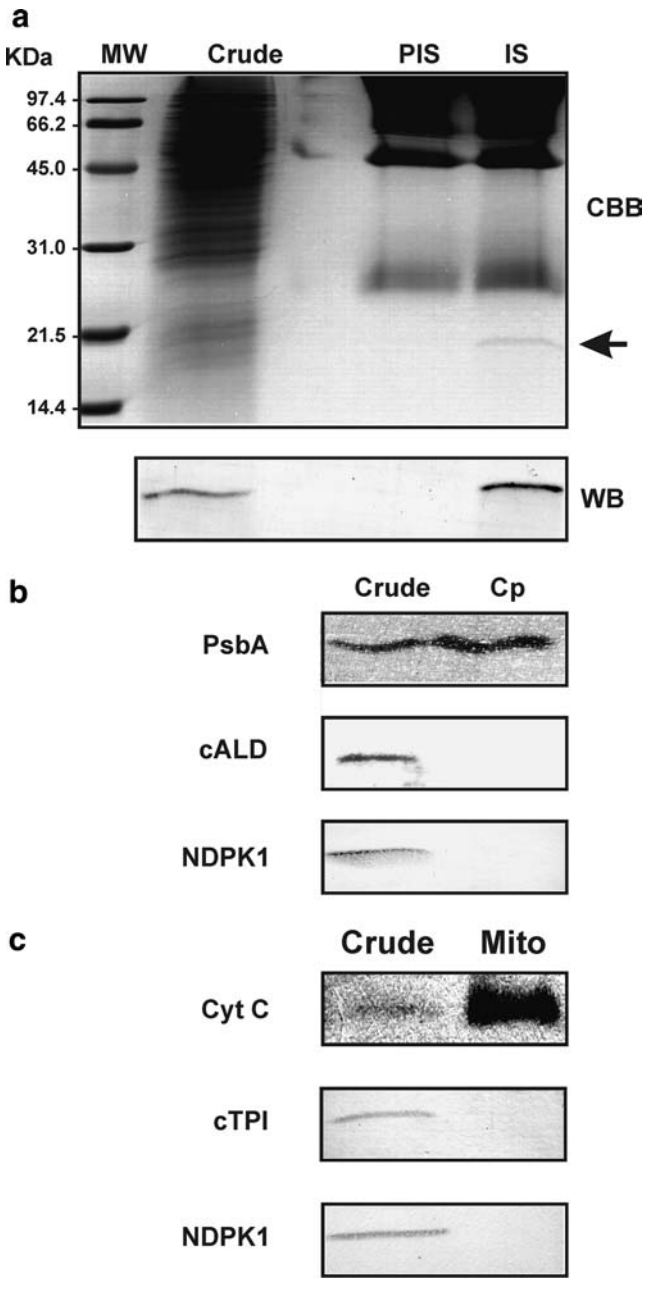


Fig. 4 Specificity of the anti-NDPK1 immune serum. **a** SDS/PAGE (*upper panel*) and immunoblot analysis (*lower panel*) of the protein immunoprecipitated with the anti-NDPK1 immune serum. *MW* molecular weight standards. Potato cells were extracted and subjected to immunoprecipitation with anti-NDPK1 immune serum (*IS*) or the corresponding pre-immune serum (*PIS*). Immunoprecipitated proteins were analysed by SDS/PAGE followed by Coomassie staining (*CBB*). An aliquot of the crude extract (15 μg protein) was also loaded on the gel to serve as reference (Crude *lane*). Two identical gels were transferred onto nitrocellulose or Bio-Rad Sequiblot membrane and subjected to an immunodetection with affinity-purified anti-NDPK1 IgGs (*WB*) or N-terminal Edman sequencing, respectively. The *arrow* on the *right* marks the position of the band detected by immunoblot. This band was cut out from the IS *lane* and further subjected to N-terminal Edman sequencing (see details in the text). **b** Anti-NDPK1 antibodies do not cross-react with polypeptides present in purified chloroplasts. Potato leaf chloroplasts were purified. Samples of crude extract (Crude) and chloroplast fraction (Cp) containing identical amounts of proteins were denatured and analysed by immunoblot using an anti-PsbA immune serum (PsbA, chloroplastic marker), anti-cytosolic aldolase affinity-purified IgGs (cALD, cytosolic marker) or the anti-NDPK1 immune serum (NDPK1). **c** Anti-NDPK1 antibodies do not cross-react with polypeptides present in washed mitochondria. Potato tuber mitochondria were purified. Samples of the crude extract (Crude) and mitochondrial fraction (Mito) containing identical amounts of proteins were denatured and analysed by immunoblot using an anti-cytochrome c mAb (cyt c, mitochondrial marker), anti-cytosolic triose phosphate isomerase (cTPI, cytosolic marker) or the anti-NDPK1 immune serum (NDPK1)

anti-NDPK1 immune serum and co-migrated with the immunoreactive band present in the crude potato cell extract (Fig. 4a, lower panel). The immunoprecipitated polypeptide was transferred onto Sequi-blot membrane, stained with Coomassie blue, cut out from the membrane and further subjected to automated N-terminal Edman sequencing. A single amino acid sequence (NH_2-MEQTFIM) was obtained, which corresponds to the consensus sequence present at the N-terminus of dicotyledon NDPK1 isoforms (Fig. 1). Thus, the polypeptide immunoprecipitated by the anti-NDPK1 immune serum corresponds to NDPK1. Its SDS-PAGE migration position in Fig. 4a (slightly above the expected molecular mass of 16.2 kDa) is probably due to the absence of reductant treatment during the preparation of these samples. These results demonstrate that the anti-NDPK1 immune serum specifically recognizes the cytosolic isoform in potato.

In order to further demonstrate the specificity of the serum towards cytosolic NDPK, we investigated the sub-cellular localization of the NDPK polypeptide detected by our antibody. Potato chloroplasts and mitochondria were purified from mature leaves and tuber, respectively. Crude, chloroplasts and mitochondria extracts were compared for their contents in immunoreactive NDPK polypeptides, PsbA (chloroplastic marker), cytochrome c (mitochondrial marker), cytosolic aldolase and triose phosphate isomerase (cytosolic markers) (Fig. 4b, c). Sub-cellular distribution of the NDPK isoform recognized by our anti-NDPK1 antibody coincided with that of the cytosolic aldolase and cytosolic triose phosphate isomerase, suggesting a cytosolic localization. No cross-reaction of the anti-NDPK1 with the chloroplastic or mitochondrial isoforms was detected. Taken together, these data strongly support the view that the immune serum generated against *S. chacoense* NDPK1 specifically recognizes the cytosolic NDPK isoform present in potato.

Total extractable NDPK activity and expression of NDPK1 in various potato tissues

Plant mitochondrial NDPK appears differentially expressed in various organs (Escobar Galvis et al. 1999). To examine the occurrence of tissue-specific expression

of NDPK1, we investigated the distribution of total NDPK activity and cytosolic NDPK expression in potato tissues. Activity measurements were performed on extracts obtained from various vegetative and reproductive tissues (Fig. 5a). Immunoblot experiments were performed on the same extracts with the anti-NDPK1 immune serum (Fig. 5b). Total NDPK activity could be clearly detected in all tissues surveyed. The lowest levels of activity were found in sepals (0.41 ± 0.06 U/mg protein), whereas the highest activity (2.05 ± 0.22 U/mg protein) was found in actively growing cell cultures (7 days after subculture). A single band with a M_r of 16.2 kDa was detected in all tissues surveyed, corresponding to the expected M_r of the polypeptide encoding NDPK1 (Fig. 5b). The immuno-reactive NDPK1 signal roughly followed the same trend as activity levels: highest in growing cell cultures and lowest in sepals. These data indicate that NDPK is found in all tissues. There is also a good correlation between total extractable NDPK activity and the intensity of the signal obtained by immunoblot analysis. This latter observation raised the possibility that NDPK1 accounts for most of the extractable NDPK activity in surveyed tissues.

Analytical purification of NDPK isoforms in various potato tissues

In order to further evaluate the importance of NDPK1 activity in relation to other NDPK isoforms in potato tissues, protein extracts were analysed by anion exchange chromatography on DEAE fractogel (Fig. 6a–d). This matrix, on which the weak exchanger DEAE is located at the end of tentacles, has been successfully used to separate and analyse isozymes of a number of metabolic enzymes (Rivoal et al. 1996; Rivoal et al. 1998; Smith et al. 2000; Dorion et al. 2005). For all the potato extracts analysed on DEAE Fractogel, overall activity recovery in chromatographic fractions was >90% and three peaks of NDPK activity were detected. A first activity peak, accounting for approximately 10% of total extractable activity in all extracts, eluted in the column flow-through (not shown). Two other NDPK peaks eluted between 150 and 250 mM KCl. The first of these two peaks always accounted for the bulk of extractable NDPK activity: from 70% of total activity in expanding leaves to about 80% of total activity in non-photosynthetic organs. It eluted around fractions 25–28 in all the samples. The second of these two peaks was more variable in relative amount (7–15% of total activity) and in retention time (between fractions 33 and 45) depending on the tissue. Fractions eluting from the columns were subjected to immunoblot analysis with the anti-NDPK1 immune serum to locate NDPK1 elution within these profiles (Fig. 6e–h). The immunodetection results were almost identical and clearly indicated that only the main activity peak contained the protein recognized by the anti-NDPK1 immune serum. Moreover, the elution profile of the 16.2 kDa NDPK1 polypeptide followed exactly the NDPK activity profile of this peak. Thus, DEAE fractogel analysis of NDPK activity in potato expanding leaves, cell cultures, root tips and tubers support the view that NDPK1 is the predominant isoform in these tissues.

NDPK1 expression is linked to the growth phase in potato cell cultures and the meristematic region in roots and shoots

Since the highest NDPK1 expression and activity levels were found in actively growing cell cultures (Fig. 5), we investigated if NDPK1 expression varied during the culture cycle in these cells and, in particular, if NDPK1 expression was linked to the growth phase of the culture. Cells were subcultured in MS media containing 2% (w/v), 0.1 % (w/v) sucrose or 0.1% (w/v) sucrose re-supplied to 2% (w/v) after 1 day of culture. Cell growth, total NDPK activity and NDPK1 protein level were monitored over the following 19 days (Fig. 7). In cultures supplied with 2% (w/v) sucrose, highest NDPK activity (Fig. 7b) and NDPK1 immuno-reactive peptides (Fig. 7c) were found during the exponential growth phase (days 4–15) of the subculture. Reducing sucrose concentration in MS media to 0.1% resulted in severe growth limitation (Fig. 7a). It must nevertheless be emphasized that these cultures continued to grow, albeit at a much lower rate, as evidenced by a small but significant gain in FW. Under sucrose limitation, the rise in total NDPK activity and NDPK1 protein did not occur. Extractable NDPK activity remained fairly stable throughout the duration of the experiment (Fig. 7b) whereas NDPK1 levels decreased (Fig. 7c). Manipulation of growth by addition of sucrose to 2% (w/v) after 1 day at 0.1% (w/v) restored cell growth after a lag (Fig. 7a). In the re-supplied cultures, NDPK extractable activity (Fig. 7b) and NDPK1 expression (Fig. 7c) were found at values between the 2% and 0.1% (w/v) treatments. These data underline that the observed correlation between NDPK1 steady-state levels and active growth of the cell culture is related to the growth status of the cells rather than culture age.

To further investigate the relationship between NDPK1 expression and plant growth, we examined NDPK activity and NDPK1 protein levels in root tips and axis. Root tips contain meristematic cells actively involved in cell divisions and can be easily dissected from the rest of the root. Young potato roots were divided in 0.5 or 1 cm segments which were numbered according to their position along the root axis (segment 1 corresponding to the root tip). Tips and segments were then extracted and assayed for NDPK activity (Fig. 8a). NDPK1 protein level was estimated by immunoblot analysis on the same extracts (Fig. 8b). Highest levels of total NDPK activity and NDPK1 expression were found in the first 5 mm of the apex, coinciding with the region of active cell division and growth. About 80% less activity was observed in segments distal from the tip.

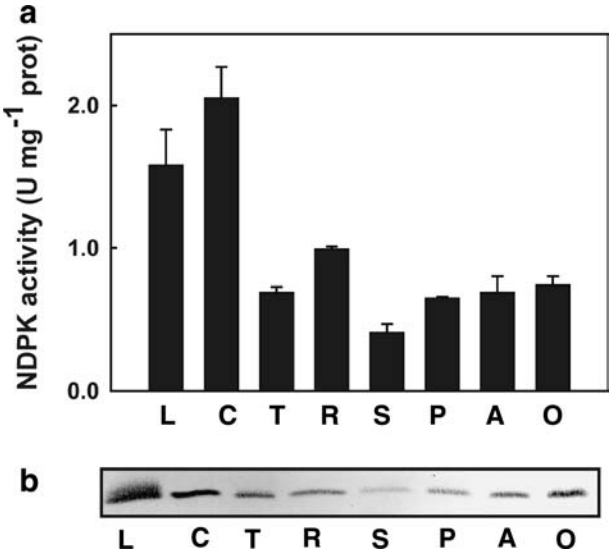


Fig. 5 Total extractable NDPK activity and NDPK1 protein levels in potato cell cultures and various potato vegetative and reproductive tissues. **a** NDPK specific activities in potato tissues and cells. Desalted extracts were obtained and NDPK activity was determined using the HPLC assay. Specific activity values are mean ± SE for at least three independent experiments. **b** Representative immunoblot analysis of NDPK1 steady-state levels in potato tissues and cells. Twelve micrograms of proteins were separated by SDS/PAGE and transferred onto a nitrocellulose membrane. Immunodetection was carried out with the anti-NDPK1 immune serum. Samples were as follows: *L* expanding leaves; *C* 7-day-old cell cultures; *T* cold-stored tubers; *R* whole roots, *S* sepals; *P* petals; *A* anthers; *O* ovaries

NDPK1 expression was also significantly reduced in the segments.

Immunolocalization of NDPK1 in roots and shoot apical meristems

The localization of NDPK1 was further examined in root and shoot meristems. The tissues were fixed, embedded, sectioned and prepared for immunocytochemistry studies (Fig. 9). The sections were incubated with the anti-NDPK1 antibody to localize the NDPK1 protein. Treatment of sections with pre-immune serum did not produce any signal in any cell type on these sections (Fig. 9a, d). Labelling was predominantly observed in the tip in longitudinal sections of root (Fig. 9b), as expected from experiments presented in Fig. 8. In more distal segments (Fig. 9e, also visible in Fig. 9b) labelling became fainter in vascular and epidermal cells. Magnified views (Fig. 9e–g) of parts of Fig. 9b revealed that labelling in the root tip was present in the meristem, provascular tissues and endoderm. The root cap contained very little NDPK1 compared to the adjacent tissues (Fig. 9b, g). A transversal section done approximately 3 mm from the root tip shows that provascular cells are the most heavily stained tissue in that region. The expression profile of NDPK1 observed in longitudinal sections of potato shoot apices revealed a similar pattern (Fig. 9h). NDPK1 protein was also highly expressed in the meristematic region, provascular tissues and shoot epidermis. These data uncover that the enzyme is highly expressed in meristematic and provascular cells, a previously unknown but important feature of plant NDPK1. A declining gradient in activity and protein level between the meristem and the mature tissues is also evident in roots.

Discussion

S. chacoense NDPK1 encodes a cytosolic NDPK isoform and the anti-NDPK1 immune serum is isoform specific

In this study, we present separate lines of evidence showing that the NDPK isolated from *S. chacoense* encodes a cytosolic NDPK isoform. First, the sequence encoded by the cDNA does not exhibit the N-terminal extension found in organellar NDPKs. It also possesses the consensus motif NH_2-MEQTFI found at the N-terminus of other dicotyledon cytosolic NDPKs (Fig. 1). Second, the protein sequence encoded by the cDNA groups with other putative plant cytosolic NDPKs (Fig. 2). Third, a polyclonal antiserum raised against the recombinant NDPK protein specifically immunoprecipitates a potato polypeptide with the sequence MEQTFI at the N-terminus. Fourth, this antiserum showed no cross-reactions with proteins present in purified chloroplasts and mitochondria. Taken as a whole our results also demonstrate that the anti-NDPK1 immune serum is specific for the cytosolic NDPK isoform found in potato.

Recombinant *S. chacoense* NDPK1 is not significantly affected by pH changes in the physiological range but appears very sensitive to the NTP/NDP ratio

Analysis of *S. chacoense* NDPK1 kinetic properties shows that the enzyme has a pH optimum in the neutral range. This is similar to observations made on purified spinach leaf isoforms (Nomura et al. 1991; Zhang et al. 1995). Remarkably, less than 10% variation in activity occurred between pH 6.5 and 8. The pH of the plant cytosol usually lies between 7.2 and 7.5 (Roberts et al. 1984a, b; Gout et al. 2001). Cytosolic pH is highly controlled but can change drastically under conditions such as oxygen deprivation (Roberts et al. 1984a; Gout et al. 2001). It is unlikely, however, that such variations in pH alone would affect the steady-state velocity of the enzyme. Calculated Michaelis–Menten constants for recombinant NDPK1 were in the same range as those of other plant NDPKs (Sommer and Song 1994; Zhang et al. 1995; Shen et al. 2005). Similar to purified spinach leaf NDPK1, recombinant *S. chacoense* NDPK1 had a higher affinity for GTP and ATP than CTP. A lower K_m value was obtained for UDP compared to TDP, simi-

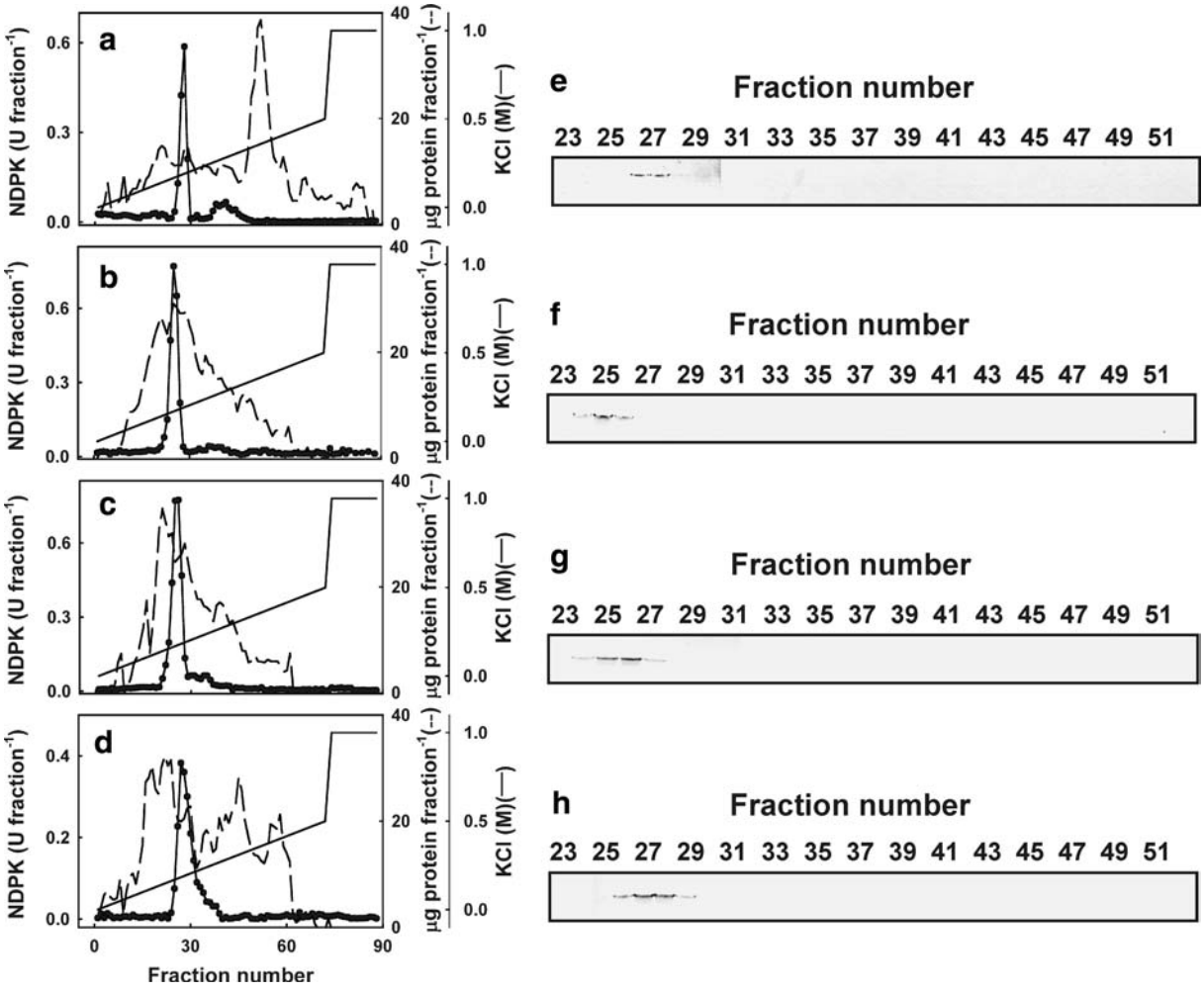


Fig. 6 Analysis of NDPK isoforms in various potato tissues using anion exchange chromatography. Potato extracts were subjected to analytical purification of NDPK isoforms using anion exchange chromatography followed by immunoblot analysis. Analyses were done on extracts prepared from expanding leaves (**a**, **e**), cell cultures (**b**, **f**), 5 mm root tips (**c**, **g**) and tubers (**d**, **h**). After desalting on PD-10 columns, 2 mg proteins were loaded on a 6 ml DEAE Fractogel column. Proteins were eluted using a KCl gradient. The elution profile of NDPK activity (**a**–**d**) (*filled circle*) is plotted as a function of fraction number (1 ml per fraction). Assay of fractions using the spectrophotometric and HPLC assays gave identical profiles. Representative immunoblots of fractions containing NDPK activity were done using the anti-NDPK1 immune serum (**e**–**h**). Fraction numbers above the blots refer to the corresponding chromatogram on the *left* side of the figure

larly to previous observations on purified spinach leaf isoforms (Zhang et al. 1995). Cytosolic concentrations of ATP and UDP have been estimated in potato tubers following non-aqueous fractionation of the tissue (respectively 0.21 mM and 0.058 mM) (Farré et al. 2001). These concentrations are comparable to NDPK1 apparent K_m values for ATP and UDP. It thus appears likely that in vivo NDPK1 activity is tightly linked to substrate availability, and consequently to respiration rates in roots. Another notable property of recombinant NDPK1 is its high sensitivity to the NTP/NDP ratio in the reaction mixture (Fig. 3c). High concentrations of acceptor relative to donor have an inhibitory effect on NDPK1 activity. Over the range of substrate concentrations tested, optimal activity occurred in the presence of an excess of 2- to 3-fold of NTP relative to NDP. In healthy plant cells, the donor to acceptor ratio is normally 3 to 10 for adenine and uridine nucleotides (Dancer et al. 1990b; Roscher et al. 1998; Farré et al. 2001). Based on these data, it is likely that root tissues in which respiratory ATP production is high (e.g. meristems, see below) provide favourable conditions for NDPK1 activity.

Cytosolic NDPK constitutes the bulk of NDPK activity in all tissues surveyed but is predominantly expressed in meristematic and provascular cells

Little information is available on the expression of plant NDPKs. To understand the relative contribution of NDPK1 to overall NDPK activity in plant tissues, we generated an immune serum against *S. chacoense* recombinant NDPK1 and took advantage of its high

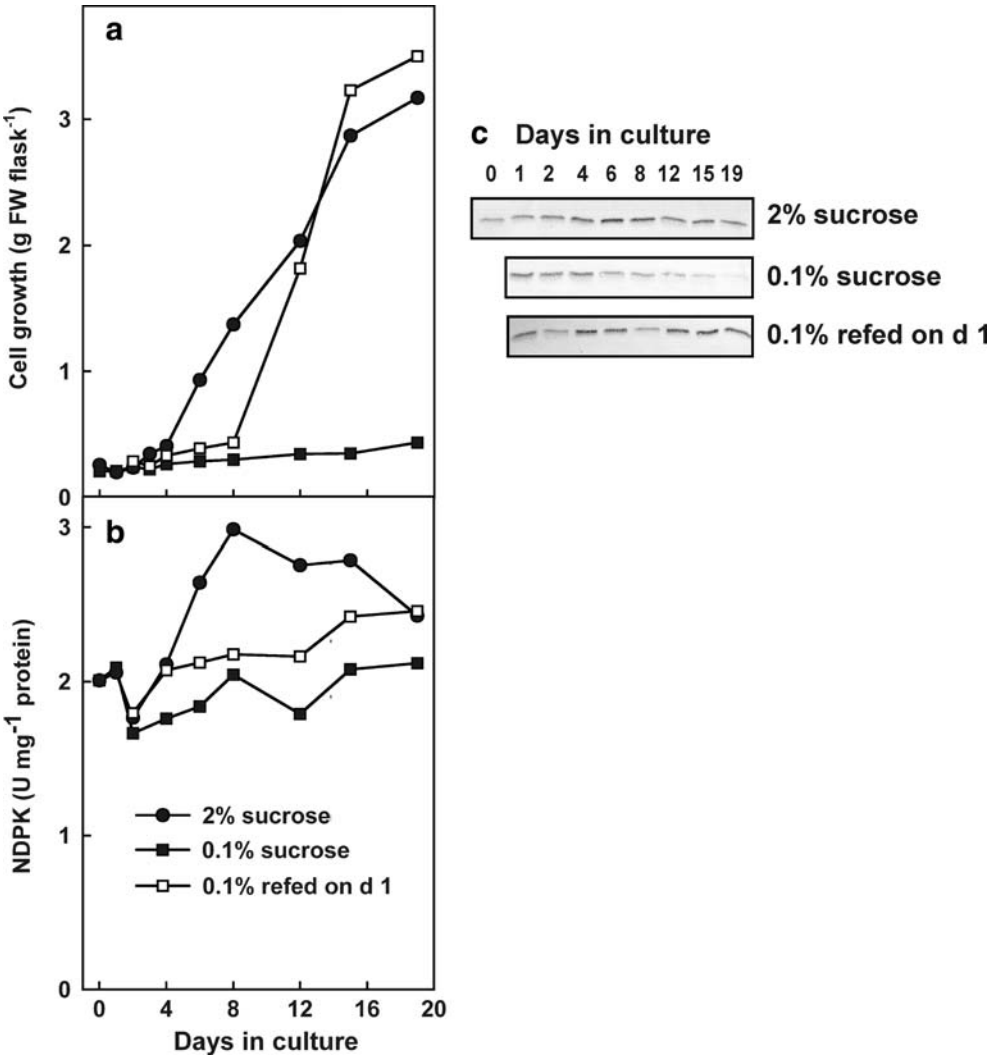


Fig. 7 Representative data for growth, NDPK activity and levels of NDPK1 protein in potato cells cultured under different sucrose regimes. Potato cells were sub-cultured in MS media containing 2% (w/v) (*filled circle*), 0.1% (w/v) sucrose (*filled square*) or 0.1% (w/v) sucrose re-supplied to 2% (w/v) after 1 day (*open square*). Cell growth was monitored over 19 days and measured as fresh weight accumulation (**a**). Total extractable NDPK activity was measured in extracts of cells harvested at different time points during subculture (**b**). Analysis of NDPK1 protein levels in extracts was done by immunoblot using the anti-NDPK1 immune serum (**c**). The experiment was repeated three times with similar results

specificity towards the potato NDPK1, as shown by immunoprecipitation, sub-cellular fractionation and immunoblot experiments (Fig. 4). This immune serum was used to uncover NDPK1 expression pattern in potato tissues and cell cultures. Analytical DEAE Fractogel chromatography of NDPK activity was performed in expanding leaves, cell cultures, root tips and tubers (Fig. 6). In these tissues, three NDPK isoforms can be separated, similarly to the situation found in spinach leaves (Nomura et al. 1991; Zhang et al. 1995). Since NDPK activity in crude extracts was very stable, our chromatographic analyses probably constitute a good assessment of the relative importance of the most abundant NDPK isoform in plant tissues. Activity profiles and immunoblot analyses of chromatographic fractions (Fig. 6) support the view that NDPK1 is the predominant isoform in all the tissues surveyed. Our estimation of the relative abundance of cytosolic NDPK (70–80% of total extractable NDPK activity) is higher than previous estimates (about 50%) made using membrane filtration and differential centrifugation techniques (Dancer et al. 1990a). The cytosolic compartment in leaf, root and tuber tissues therefore contain the highest potential for NTP synthesis from ATP. This is consistent with estimates of sub-cellular distribution of UTP, which is predominantly localized in the cytosol in spinach leaves and potato tubers (Dancer et al. 1990a; Farré et al. 2001). NDPK activity was present in all the tissues surveyed. Likewise, judging from immunoblot analysis (Fig. 5), NDPK1 was clearly detectable in all tissues, perhaps with the exception of sepals. This latter finding is consistent with a RT-PCR study of NDPK1 expression pattern in *Brassica campestris* flower organs (Shin et al. 2004). In potato, NDPK activity and

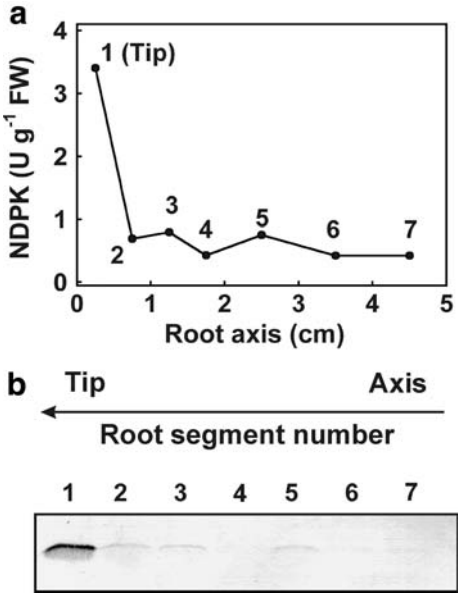


Fig. 8 NDPK activity and NDPK1 protein levels in potato roots. Potato roots were divided in 0.5 or 1 cm segments. Segments were numbered and harvested separately according to their position along the root axis (segment 1 corresponded to the root tip). Batches of root segments were then assayed for NDPK activity. Activity was plotted as a function of the segment's position along the root (**a**). The extracts were also subjected to immunoblot analysis using the anti-NDPK1 immune serum (**b**)

NDPK1 protein levels were comparable in male and female flower parts (Fig. 5). *B. campestris* NDPK mRNAs were undetectable in stamens and BcNDPK1 was only found in pistils (Shin et al. 2004). These data suggest different expression patterns between the two plants or regulation of expression at the translation level. Total NDPK activity and NDPK1 protein were found at a maximum during the logarithmic growth phase of potato cell cultures (Fig. 7). This feature was not associated with the age of the culture, but rather with its ability to grow actively as shown by sucrose limitation and re-feeding studies. It has been demonstrated that sucrose stimulates cell division in plants through regulation of D-type cyclin genes (Riou-Khamlichi et al. 2000). Sucrose availability is also known to control cellular respiration rate (Contento et al. 2004). Hence, the manipulation of sucrose availability has consequences on a large number of cellular processes including energy metabolism (Contento et al. 2004). Interestingly, in sucrose-limited cells, total NDPK extractable activity remained fairly constant whereas NDPK1 levels declined steadily with time. We interpret these data as evidence that some compensation occurs between NDPK1 and other NDPK isoforms in sucrose limited cells. Our results do not allow us to conclude if NDPK1 expression is directly under the control of sucrose availability. We can, however, deduce that NDPK1 expression is linked to the capacity of cell cultures to grow. In this context, the relationship between NDPK1 expression and nutrient status deserves further investigation. Experiments carried out *in planta* provided further evidence of a link between NDPK1 expression and growth. Indeed, analysis of NDPK activity and NDPK1 expression in dissected root segments showed high levels of NDPK1 in the tip compared to the root axis (Fig. 8). Furthermore, immunolocalization of NDPK1 showed that the protein is found in the meristematic region in roots and shoots (Fig. 9). Data gathered from DNA microarray expression analysis of poplar wood forming meristems (Hertzberg et al. 2001; Schrader et al. 2004) are consistent with our conclusions. Indeed, hybrid aspen NDPK1 EST (GenBank accession number AI163635) is more highly expressed in the cambial meristem undergoing cell division, expansion and elongation (Hertzberg et al. 2001) compared to more mature cambium. These data are also reminiscent of studies that point to a link between NDPK and growth in plants and non-plant NDPKs. In rice seedlings transformed with a heat-inducible antisense NDPK1 construct, epidermal cell elongation in the coleoptile was reduced (Pan et al. 2000). In animals, enhanced *NDPK/NM23* gene expression often takes place in conjunction with enhanced cell growth. For example, in rat embryo fibroblasts microinjected with an antibody specific to NM23, cell division was inhibited (Sorscher et al. 1993). It is, however, apparent from other studies that isoforms NM23-H1 and NM23-H2 may have different effects on growth depending on the cell type considered (Kimura et al. 2000). Our work provides for the first time direct evidence that the highest levels of NDPK1 activity are found in meristematic regions in plants. We also show that NDPK1 was found in root and shoot provascular tissues. Thus, NDPK1 localization supports the view that this protein plays a role in plant differentiation as well as growth. It is important to note that the distribution of NDPK1 is also consistent with a housekeeping function since it is present in all the tissues that were surveyed in the present work.

Metabolic implications of the localization of NDPK1 in meristems and provascular tissues

It has been proposed that NDPK plays a significant role in the equilibration of ATP and UTP pools in plants and other systems (Dancer et al. 1990a; Lambeth et al. 1997; Roberts et al. 1997). Evidence for this comes from the observation that ATP/ADP and UTP/UDP ratios vary in a relatively similar manner in plant tissues subjected to uncouplers of oxidative phosphorylation, Pi stress or O_2 deprivation (Dancer et al. 1990b; Rolletschek et al. 2003; van Dongen et al. 2004). In non-photosynthetic tissues, ATP production is coupled to respiration. Root O_2 consumption rates are the highest in the meristem, in particular in the cell division and expansion zones (Bidel et al. 2000; Mancuso and Boselli 2002). Consequently, these tissues are likely to contain high NTP/NDP ratios and are expected to provide favourable conditions for

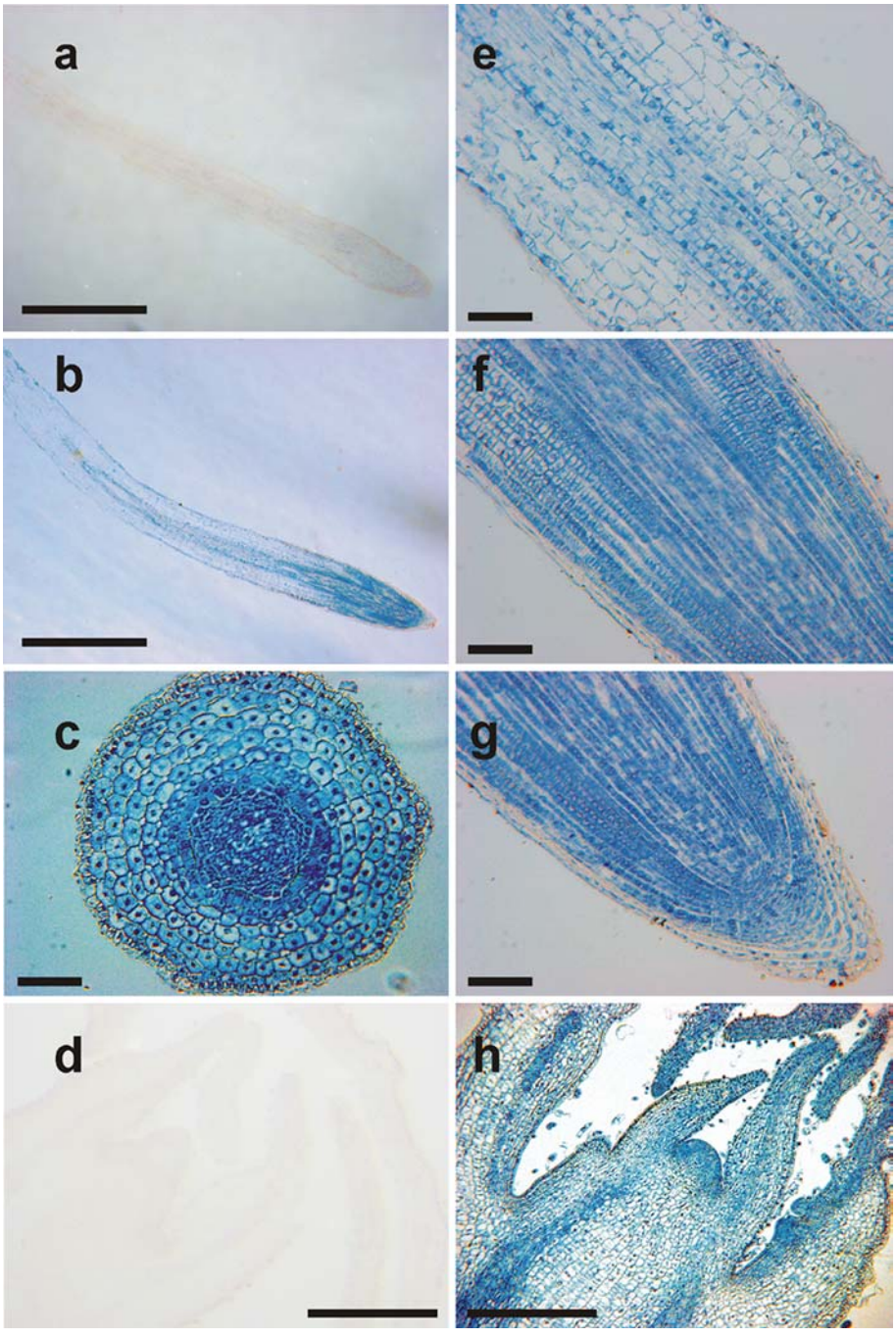


Fig. 9 Immunolocalization of NDPK1 in potato root and shoot meristems. Immunolocalization was done on longitudinal (**a**, **b**, **d**, **e**, **f**, **g**, **h**) or transversal (**c**) sections of root apical region (**a**, **b**, **c**, **f**, **g**), root distal segments (**e**) or apical shoot apex (**d**, **h**) with pre-immune serum (**a**, **d**) or anti-NDPK1 immune serum (**b**, **c**, **e**, **f**, **g**, **h**). Sections **e**, **f** and **g** represent higher magnification of the regions, respectively, located approximately 1 cm from the tip, 3 mm from the tip and at the tip. Section **c** is taken approximately 3 mm from the tip. *Scale bars* are 5 mm (**a**, **b**), 400 μm (**c**, **e**, **f**, **g**) and 1 mm (**d**, **h**)

directional phosphoryl transfer from ATP to NDPs (e.g. net synthesis of NTP) by NDPK1.

In the meristematic region, NTPs are needed as precursors to sustain two important biosynthetic pathways: the first is the synthesis of nucleic acids (e.g. replication of DNA). However, the absolute flux of NTPs towards RNA and DNA synthesis is relatively minor in comparison to global cell C budget. In actively growing tomato cell cultures, it has been estimated that nucleic acids synthesis consume at most 0.1% of the glucose metabolized (Rontein et al. 2002). The other pathway that heavily depends on NTPs is the synthesis of precursors for the cell wall. UTP, in particular, is required for the synthesis of the diverse family of UDP-sugars by UDP-sugar pyrophosphorylases as well as for the synthesis of UDP-glucuronic acid by UDP-glucuronic acid pyrophosphorylase (Reiter and Vanzin 2001; Seifert 2004). The gateway to this family of metabolites is UDP-Glc, which can be formed by sucrose synthase (EC 2.4.1.13) and UDP-Glc pyrophosphorylase (EC 2.7.9.9). Biochemical activity assays and in situ activity staining of sucrose synthase in wheat roots showed that the activity of this enzyme was slightly lower in the apex compared to distal seg-

ments (Albrecht and Mustroph 2003). ^{31}P NMR studies in maize root tips have shown that the in vivo flux of UDP-Glc pyrophosphorylase towards UDP-Glc synthesis is very high, with calculated values of 0.6–4 μmol min^{-1} g^{-1} FW (Roberts 1990; Roscher et al. 1998). Hence, if the flux towards UDP-Glc has a comparable value in potato, extractable levels of NDPK1 in potato root tips (~3.5 μmol min^{-1} g^{-1} FW) can account for the large amounts of UTP consumed by UDP-Glc pyrophosphorylase. It is worth emphasizing that in the coupled NDPK/UDP-Glc pyrophosphorylase reaction, the second reaction recycles energy as it generates PPi which can be used by pyrophosphate:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90) to support the net glycolytic flux catalysed by this enzyme (Hajirezaei et al. 1994; Roscher et al. 1998). The expression of NDPK1 in root tips is not without reminding that of *A. thaliana* UDP-Glc dehydrogenase (EC 1.1.1.22), which catalyzes the conversion of UDP-Glc to UDP-glucuronic acid (Seitz et al. 2000). NDPK1 could thus function in primary cell wall synthesis by providing UTP needed for UDP-Glc synthesis in the cytosol. This function would be consistent with the observed tissue expression pattern of NDPK1 because primary cell wall is normally laid out during cell division and wall material deposition continues with the maturation of the cell. Provascular tissues are sites of intense cell wall synthesis that must have a steady supply of UTP to form UDP-Glc by UDP-Glc pyrophosphorylase. NDPK, however, is not the only enzyme capable of producing UTP and other NTPs, since pyruvate kinase (EC 2.7.1.40) can phosphorylate NDPs, albeit at a lower efficiency than ADP (Turner and Plaxton 2000). Since the two enzymes coexist in the cytosol, it remains to be seen how each of them affect NTP metabolism in plants. It should finally be emphasized that it is still unknown if plant NDPKs, including NDPK1, carry alternate biochemical activities, as do their animal counterparts. The distinct expression pattern of NDPK1 should then be taken into account when evaluating the impact of such activities.

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