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ORIGINAL PAPER

Heat shock modulates phosphorylation status and activity of nucleoside diphosphate kinase in cultured sugarcane cells

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Abstract Nucleoside diphosphate kinase (NDPK) is involved in the regeneration of nucleoside triphosphates (NTPs) through its phosphotransferase activity via an autophosphorylating histidine residue. Additionally, autophosphorylation of serine and/or threonine residues is documented for NDPKs from various organisms. However, the metabolic significance of serine/threonine phosphorylation has not been well characterized. In this study we report the cloning and characterization of NDPKI from cultured sugarcane (*Saccharum officinarum* L. line H50-7209) cells, and modulation of serine autophosphorylation of NDPK1 in response to heat-shock (HS). Heat-shock treatment at 40°C for 2 h resulted in a 40% reduction in labeled phosphoserine in NDPK1. This dephosphorylation was accompanied by an increase in NDPK enzyme activity. In contrast, NDPK1 in cultured tobacco (cv. W-38) cells did not show changes in autophosphorylation or increased enzyme activity in response to HS. The mRNA or protein level of NDPK1 did not increase in response to HS. Sugarcane cells sustain the constitutive protein synthesis in addition to heat-shock protein synthesis during HS, while constitutive protein synthesis is significantly reduced in tobacco cells during HS. Thus, HS modulation of NDPK1 activity and serine dephosphorylation in sugarcane cells may represent an important physiological role in maintaining cellular metabolic functions during heat stress.

Keywords Sugarcane · *Saccharum officinarum* · Heat-shock · Nucleoside diphosphate kinase · Serine phosphorylation

Abbreviations

NDP	Nucleoside diphosphate
HS	Heat shock
HSP	Heat-shock protein
HSR	Heat shock response

Introduction

Nucleoside diphosphate kinase (abbreviated NDPK or NDK) (EC 2.7.4.6) is a ubiquitous enzyme that catalyzes the conversion of nucleoside diphosphates (NDPs) into nucleoside triphosphates (NTPs) through a ping-pong mechanism (Parks and Agarwal 1973). The reaction is mediated by a conserved histidine residue on the NDPK enzyme, and serves as the phosphorylated intermediate (Morera et al. 1995). In contrast to the early concepts of NDPK as a general housekeeping enzyme, later studies suggest physiologically essential multi-functional role for this enzyme (reviewed in Mehta and Orchard 2009). NDPKs associate with different sub-cellular structures, and form complexes with other proteins, as well as with nucleic acids (Thakur et al. 2009; Kavanaugh-Black et al. 1994; Engel et al. 1998; Galvis et al. 2001). The NDPK isoforms associated with chloroplasts, mitochondria, cytosol, plasma

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membrane and microtubules suggest widespread regulatory roles in cellular metabolism involving generation of non-adenine nucleotides, G-protein activation, and cell motility either directly or indirectly (Wieland 2007; Hippe et al. 2003). NDP kinases have been studied in more detail in animal systems due to their potential role in metastasis suppression and functions related to human health (reviewed in Mehta and Orchard 2009). Based on current understanding of animal NDP kinases, their functions have been divided into three major groups. They are (1) nucleoside diphosphate kinase activity, (2) protein histidine kinase activity and (3) $3' \rightarrow 5'$ DNA Exonuclease activity associated with DNA binding and proof-reading ability (Mehta and Orchard 2009).

In light of the apparently essential roles of NDPKs in cellular metabolism, growth and development, the regulation of expression and activity of NDPKs in response to environmental signals in plants has not been studied in detail. Involvement of different NDPK2 isoforms in phytochrome signaling (Choi et al. 1999; Genoud et al. 2008), auxin responses (Choi et al. 2005), and coleoptile elongation (Pan et al. 2000) have been reported. Further, Shen et al. (2008) demonstrate a function for NDPK2 in activating GTPase in Arabidopsis, and suggest that NDPK2 may be a missing link between phytochrome mediated signaling and G-protein mediated signaling. Transcript levels of NDPK isoforms are increased in response to wounding in tomato (Harris et al. 1994) and in response to glyphosate treatment in rice (Ahsan et al. 2008) indicating a stress response mechanism involved in *NDPK* gene expression. In Arabidopsis, NDPK2 isoform associates with H_2O_2 stress regulated MAP kinases (AtMPK3 and AtMPK6), and seem to increase the ability of the plant to tolerate multiple abiotic stresses (Moon et al. 2003). NDPK1 isoforms interact with catalases in both Arabidopsis and in *Neurospora crassa*, thus also been linked to ROS signaling in a wide range of organisms (Fukamatsu et al. 2003; Lee et al. 2009). A mitochondrial NDPK isoform from pea associates with a HS induced protein of unknown function (Galvis et al. 2001) suggesting a potential regulatory role for mitochondrial NDPK during HS.

In addition to the reactive phosphohistidine intermediates that perform phosphotransfer function for its nucleoside diphosphate kinase activity and protein histidine kinase activity, NDPKs undergo phosphorylation at other residues. Several studies demonstrate autophosphorylation of serine as a result of inter- or intra-molecular phosphate transfer from the phosphohistidine enzyme intermediate (Munoz-Dorado et al. 1993; Engel et al. 1998). In Nm23-H1, the human NDPK homolog, serine phosphorylation of NDPK correlates with metastasis suppressor properties (MacDonald et al. 1993). Dorion et al. (2006) demonstrated the autophosphorylation of serine 117 of the cytosolic NDPK in *Solanum* sp., and that this autophosphorylation is affected by high ADP concentrations. Replacing the serine 117 with an acidic amino acid drastically reduced the catalytic activity of the enzyme, suggesting an important physiological role for serine phosphorylation for NDPK function. The functional significance of this phosphorylation is still largely unknown.

Previous studies indicate that in contrast to many organisms, cultured sugarcane cells respond differently to HS by continuing to synthesize a full spectrum of proteins in addition to synthesizing heat-shock proteins (Moisyadi and Harrington 1989). Thus, the net protein synthesis in sugarcane cells increases during HS. Cooper and Ho (1983) documented a similar phenomenon in corn in which a new sub-set of heat-shock proteins were induced, while the synthesis of normal protein expressed at 25°C continued to produce when the temperature was increased to 40°C. On the other hand, cultured tobacco cells show a more typical heat shock response with the dramatic suppression of constitutive protein synthesis with the onset of heat shock protein synthesis (Kanabus et al. 1984; Harrington and Alm 1988). These observations suggest that a specific physiological mechanism operating in sugarcane and maize cells, and possibly in some other plant species, may rescue the normal protein synthesis at HS temperatures, pinpointing an agriculturally important aspect in plant stress responses.

NDPK function is directly involved in the generation of GTP, an essential energy source for protein synthesis, and is involved in signal transduction processes through association with G-proteins. However, the behavior of the NDPK enzyme in sugarcane and tobacco during HS has not been studied previously. The relationship between the regulation of NDPK and the atypical HS response in cultured sugarcane cells may provide an important system to study the possible involvement of this enzyme in stress tolerance mechanisms.

The goal of the work presented here was to determine the effect of HS on the expression and phosphorylation of NDPK1 in sugarcane cells. NDPK1 cDNAs were cloned and characterized in an attempt to analyze the protein sequences of different isoforms of the enzyme, and to study the expression of NDPK in response to HS. According to a previous study, NDPK in total protein extracts from heat-shocked sugarcane cells show enhanced ability to autophosphorylate at serine residues after SDS-PAGE followed by protein renaturation (Moisyadi et al. 1994). In this study we attempt to understand the basis of this enhanced phosphorylatability of NDPK after heat-shock. Further, autophosphorylation and enzyme activity of NDPK in heat-shocked sugarcane cells were compared with that in heat-shocked tobacco cells to gain an insight on the modulation of NDPK in these two systems during HS. The present

study revealed that exposure of sugarcane cells, but not of tobacco cells, to sub-lethal heat shock temperatures resulted in serine dephosphorylation that is reflected as an enhanced ability of the protein to autophosphorylate in gel, and increased enzyme activity of NDPK.

Materials and methods

Plant materials

Sugarcane (*Saccharum officinarum* L. cv. H50-7209) and tobacco (*Nicotiana tabacum* L. var. Wisconsin-38) cells were grown in the dark as suspension cultures on a rotary shaker at 25–26°C as described previously (Moisyadi and Harrington 1989; Harrington and Alm 1988). Mid log phase cultures of sugarcane cells (14 days old) and tobacco cells (7 days old) were used in all experimental procedures unless specified.

Isolation of sugarcane NDPK cDNAs

A primary cDNA library of cultured sugarcane cells, constructed in *λgt11* (Stratagene) was obtained from Dr. Paul A. Moore of Hawaii Sugar Planters Association Research Center, Aiea, Hawaii. A 627 bp cDNA containing the entire coding region of the spinach NDPK I (Nomura et al. 1992) was obtained from Dr. Atsushi Ichikawa of Kyoto University, Japan. An internal *Sau*3AI fragment corresponding to 414 bp of spinach NDPK I cDNA (genebank accession no: D10659, position 54-468) from the open reading frame was used as the probe to screen approximately 1.2×10^6 plaques. Out of 21 positive clones, two independent inserts were subcloned into the *Eco*RI site of pBluescript vector (Stratagene) and sequenced.

Heat shock treatments and protein extraction

Sugarcane and tobacco cells were incubated at temperatures ranging from 25 to 40°C for 2 h in an orbital shaking water bath. Cells (0.25 g fresh weight) were ground in liquid N_2, and extracted into 1 mL of SDS buffer as described (Moisyadi and Harrington 1989). Concentration of the proteins in the samples was assayed according to the method of Lowry et al. (1951). Twenty micrograms of total proteins was separated by single dimensional SDS-PAGE on minigels (12.5%) as described by Moisyadi and Harrington (1989). For two-dimensional IEF/SDS-PAGE, protein was extracted from control (25°C) and heat-shocked (40°C) sugarcane cells according to the phenol partition method described by Hurkman and Tanaka (1987). Fifty micrograms of total proteins was separated on minigels as described previously (Moisyadi and Harrington 1989).

In situ autophosphorylation of sugarcane and tobacco proteins

Total protein (20 μg) was separated on SDS-polyacrylamide gel (12.5%) containing 20 μg mL^{-1} Histone H1 type IIIS (Sigma Chemical Co) in the matrix to minimize the leakage of NDPK1. After electrophoresis, the proteins were renatured and *in-gel* phosphorylated using [γ-^{32}P] ATP as described before (renaturation and phosphorylation method A, Moisyadi et al. 1994). The gels were exposed to Fuji X-ray film, and bands corresponding to the 16.5–18 kDa region were excised into 3 mL of ScintiVerse E (Fisher Scientific Inc.) to quantify the incorporation of radioactivity using liquid scintillation spectroscopy.

Northern hybridization

Total RNAs were extracted from sugarcane cells from each treatment (see heat shock treatments above) according to the method of McGookin (1984). Northern hybridization was carried out with sugarcane NDPK I cDNA, SoNDPK1 (742 bp) probe using standard methods.

Western hybridization

After separation by single dimensional or two-dimensional gel electrophoresis, proteins were transferred to PVDF membrane. The blots were exposed to a primary antibody (spinach NDPKI polyclonal rabbit antisera were provided by Dr. Atsushi Ichikawa of Kyoto University, Japan), and detected using alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Sigma Chemical Co).

Immunoprecipitation of de novo ^{35}S-labeled sugarcane NDPK

Sugarcane cells (0.25 g fresh weight in 1 mL culture medium) were transferred into sterile 50 mL tubes and placed at 25°C (control) or 40°C (HS) in a shaking water bath. After 5 min, 800 μCi of Trans-^{35}S-label (ICN Biomedicals) was added to each tube and incubated for further 2 h. At the end of treatments the cells were filtered, washed thoroughly and ground in liquid N_2. Total protein was extracted into 0.5 mL of ice cold lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 5 μg mL^{-1} aprotinin and 0.5 μg mL^{-1} leupeptin, and NDPK was precipitated by spinach NDPKI polyclonal antisera. Immunoprecipitated proteins from each sample were divided into three equal aliquots and separated by SDS-PAGE. For one set of

samples, ^{35}S labeled proteins were visualized by fluorography. Alternatively, total NDPK1 (^{35}S-labelled and unlabeled) in immunoprecipitated samples were detected by western blotting using spinach NDPK antibody. Finally, the third set of immunoprecipitated samples was subjected to *in-gel* autophosphorylation. The total protocol was conducted in duplicate.

Acid stable phosphoamino acid analysis

Approximately 0.1 g of sugarcane cells in 0.5 mL culture medium were pre-incubated with 0.5 mCi of [^{32}P]-orthophosphate (New England Nuclear) at 25°C for 20 h. The cells were then washed, resuspended in 0.5 mL of fresh growth medium and subjected to 25°C (control) and 40°C (HS) for 2 h. Each treatment was done in duplicate, and total proteins were extracted. NDPK was immunoprecipitated with spinach NDPKI antibody as described above, separated by SDS-PAGE, transferred onto PVDF membranes and visualized with Fuji RX film. Bands corresponding to 16.5 kDa were excised and hydrolyzed in 5.7 N HCl, and the phosphorylated amino acid residue was identified according to the method described by Crow et al. (1990). Briefly, the hydrolysate was mixed with unlabelled phosphoaminoacid standards (P-Ser, P-Thr, P-tyr at 2 mg/ml each) and subjected to two dimensional electrophoresis on TLC plates. After electrophoresis, the phosphoamino-acid standards were localized by ninhydrin staining, and dry TLC plate was exposed to Kodak XAR5 X-ray film.

Analysis of NDPK enzyme activities

Sugarcane and tobacco cells were incubated at 25°C (control) and 40°C (HS) for 2 h, and 3 g of cells were harvested by filtration. The cells were extracted for 1 h into 6 mL of ice-cold extraction buffer (10 mM Tris–HCl pH 8, 2 mM EDTA, 2 mM EGTA, 1% β-mercaptoethanol, 0.1% CHAPS, 1 mM PMSF) with constant stirring and centrifuged at 12,000*g* for 20 min at 1°C. The supernatants were loaded onto 5 mL DEAE-Sepharose (Sigma Chemical Co) columns using gravity and eluted sequentially with 10 mL each of elution buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF) containing 0.1, 0.2, 0.4 and 0.8 M NaCl. The fractions containing 16.5 kDa autophosphorylation activity were dialyzed against 10 mM ammonium bicarbonate, lyophilized and resuspended in 100 mL of Millipore filtered H_2O. Total protein concentrations were estimated according to Lowry et al. (1951). Aliquots containing 1 µg of protein from each sample were assayed for NDPK activity as previously described (Moisyadi et al. 1994). Treatments were done in duplicate, and three independent assays were done for each sample.

Results

NDPK1 cDNAs and deduced amino acid sequences

Both cDNA clones sequenced in this study contained the entire coding region of putative sugarcane NDPK1. The SoNDPK1 (*Sacchurum officinarum* NDPK1) cDNA coded for a deduced 149 amino acid polypeptide with a predicted molecular mass of 16,415 Da (p*I* 6.81). The second cDNA (SoNDPK1.1) showed approximately 97% homology to the SoNDPK1 cDNA, especially in the coding region. However, the last codon in SoNDPK1.1 is substituted by a stop codon, resulting in the lack of a terminal lysine residue in the predicted polypeptide sequence (Fig. 1). This single amino acid change lowered the predicted isoelectric point of this isoform to p*I* 6.28 as compared to the p*I* 6.81 in SoNDPK1. The predicted molecular masses of both isoforms are in agreement with the purified sugarcane NDPK1 resolved at about 16.5 kDa upon SDS-PAGE, and may explain the occurrence of multiple isoforms of NDPK1 with a range of isoelectric points as resolved on two-dimensional gels (Moisyadi et al. 1994).

The region involved in NDPK catalytic activity was analyzed on the primary amino acid sequence deduced from SoNDPK1. The deduced amino acid sequence showed a very high sequence identity with known NDPKs (Fig. 1). The proposed catalytic site amino acid sequence 'N-X-X–H-G-S-D-(S/A)' is conserved in all known NDPKs (Gilles et al. 1991). The Prosite domain analysis program recognized the region 112-120 with amino acids 'N-v-i-H–D-S-D-S-i' as the NDPK catalytic site in the deduced sugarcane NDPK1 amino acid sequence. Additional domains identified by the Prosite analysis include two putative Casein Kinase phosphorylation sites, a putative tyrosine kinase phosphorylation site, three putative protein kinase C phosphorylation sites, and an ATP/GTP binding site (P-loop) (Fig. 1).

Autophosphorylation of NDPK is altered during HS in cultured sugarcane cells

Results of the *in-gel* phosphorylation of proteins extracted from cultured sugarcane and tobacco cells treated at different temperatures are given in Fig. 2. A minimum of 36°C heat shock was required to observe a change in phosphorylation level in sugarcane cells (Fig. 2a, f). Incorporation of labeled γ-phosphate group from ATP into sugarcane NDPK increased approximately threefold in 40°C HS treated samples compared to 25°C control (Fig. 2f). Increasing incubation temperature of cells before protein extraction correlated with increasing *in-gel* autophosphorylation of NDPK (Fig. 2f). In contrast, tobacco NDPK did not exhibit an increase in autophosphorylation in response to HS (Fig. 2b, f).

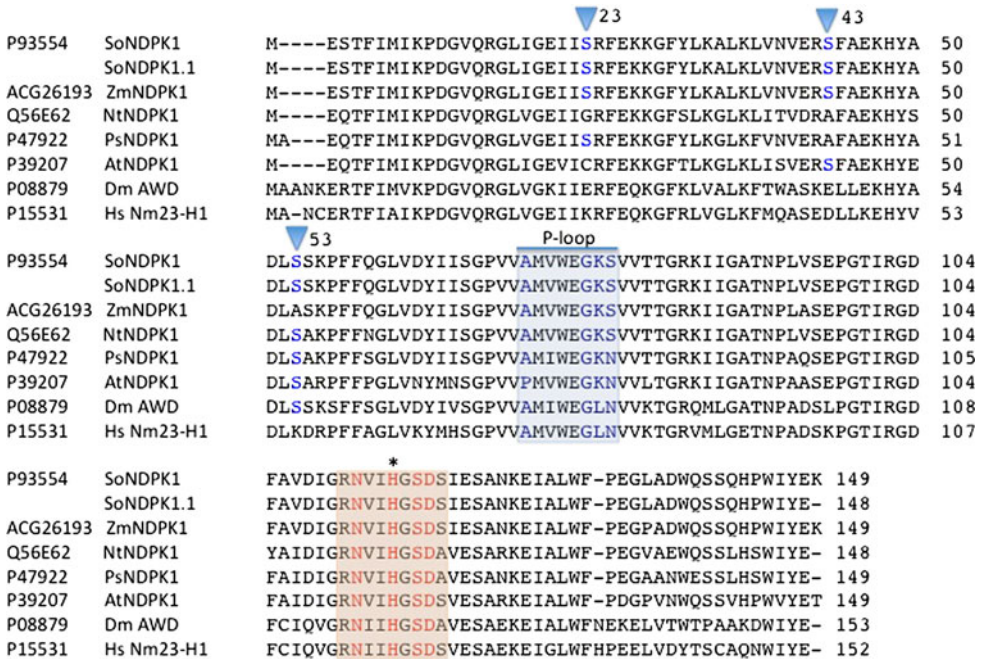

Fig. 1 Alignment of deduced amino acid sequence of SoNDPK1 clone with known NDPKs. *So Sacchurum officinarum*, *Zm Zea mays*, *Nt Nicotiana tabacum*, *Ps Pisum sativum*, *At Arabidopsis thaliana*, *Dm Drosophila melanogaster*, *Hs Homo sapiens*. *Downward triangles* indicate predicted serine phosphorylation sites. *P-loop* represents the predicted ATP/GTP binding site, and the *highlighted RNVIRHGSDS sequence* indicates the conserved NDP kinase active site. The reactive histidine residue involved in phosphotransfer activity is indicated by *asterisk*

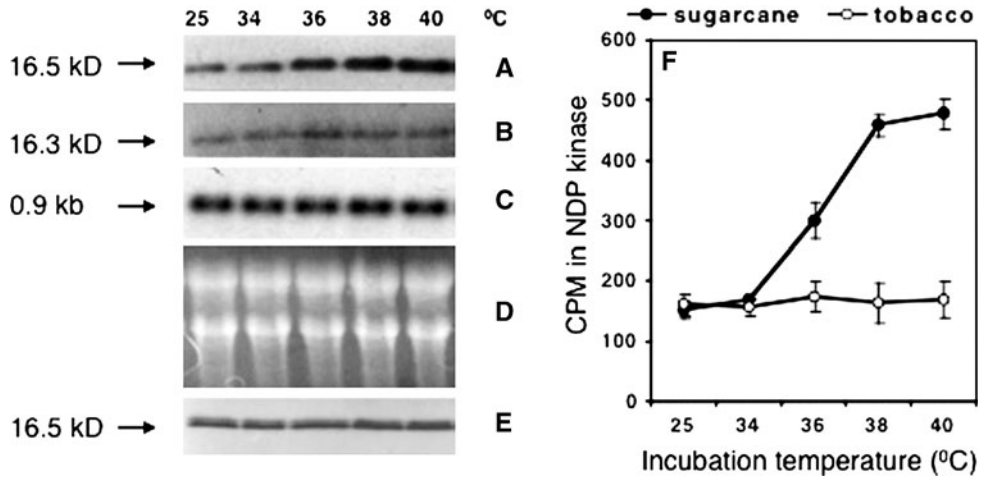

Fig. 2 Effect of heat-shock on *in-gel* autophosphorylation activity and RNA levels of NDPK. *A* autoradiograph of sugarcane proteins *in-gel* phosphorylated with ^{32}P-labeled ATP. *B* autoradiograph of tobacco proteins *in-gel* phosphorylated with ^{32}P-labeled ATP. *C* northern hybridization of total RNA (10 μg/lane) isolated from sugarcane cells heat-shocked at indicated temperatures for 2 h. The blot was probed with SoNDPK1 cDNA. *D* ethidium bromide stained RNA gel used for northern hybridization. *E* immunoblot of sugarcane total proteins (20 μg/lane) with spinach NDPK1 polyclonal antibodies. *F* analysis of 16–17 kDa region of the phosphorylated gels by liquid scintillation spectrometry. The cells were heat-shocked at indicated temperatures for 2 h prior to protein extraction. For *in-gel* autophosphorylation and immunoblotting, total protein extracts (20 μg/lane) were separated by SDS-PAGE

HS does not increase NDPK mRNA and protein levels

The results of the northern hybridization revealed that the mRNA level for NDPK1 in cultured sugarcane cells did not change considerably in response to different HS temperatures ranging from 25 to 40°C (Fig. 2c). Thus, gradually increased *in-gel* autophosphorylation of NDPK after SDS-PAGE with increasing incubation temperature did not correlate with an increased transcription from *NDPkinase* gene.

Protein extracts obtained from cells treated at the above temperatures were analyzed for NDPK levels by immunoblotting with spinach NDPK1 antibody (Fig. 2e). Protein sequence analyses revealed more than 90% identity between spinach and sugarcane proteins, and the spinach NDPK1 antibody specifically reacted with purified

sugarcane NDPK1 protein, justifying the use of spinach NDPK1 antibody for our experiments. When used with total protein extracts, the antibodies reacted strongly with a major polypeptide band at 16.5 kDa with a weak signal at 18 kDa indicating the presence of NDPK1 and NDPK2 in these cells. In repeated experiments, the levels of both NDPK1 and NDPK2 proteins did not increase in response to HS (Fig. 2e). To further analyze the levels of different NDPK isoforms, proteins extracted from cells treated at 25 and 40°C were separated by two dimensional IEF/SDS-PAGE, and immunoblotted with spinach NDPKI antibody. No major qualitative and quantitative changes in the levels of NDPK isoforms were detected in either treatment (data not shown). Thus, the increased autophosphorylation shown in Fig. 2a and f was apparently not due to increased enzyme levels.

De novo synthesis of NDPK does not increase in response to HS

In vivo ^{35}S labeling and immunoprecipitation indicated that de novo synthesis of NDPK did not increase in response to a 2 h HS at 40°C (Fig. 3a). Western hybridization with NDPK1 antibodies indicated that the total NDPK1 protein level was not increased in the HS sample, (Fig. 3b) supporting previous results shown in Fig. 2e. When these immunoprecipitated proteins were autophosphorylated in gels, the 16.5 kDa band in the HS sample showed a considerably higher intensity over the control sample (Fig. 3c) further confirming that increased enzyme synthesis was not a determining factor for the observed high autophosphorylation levels after HS treatment. This observation firmly suggests that post-translational events are responsible for the increased ability of NDPK in sugarcane cells to autophosphorylate.

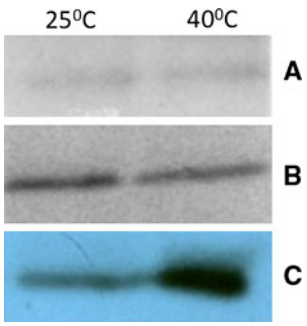

Fig. 3 Immunoprecipitation of NDPK from in vitro ^{35}S-labeled sugarcane cell extracts show no difference in de novo synthesis. *A* fluorograph of immunoprecipitated ^{35}S-labeled proteins. *B* immunoblotting of precipitated protein used in *panel A*, with spinach NDPK I antibodies. *C in-gel* autophosphorylation of immunoprecipitated proteins used in *panels A* and *B*. The cells were incubated for 2 h at temperatures indicated above each lane, before extracting proteins

Heat shock affects in vivo serine dephosphorylation in NDPK

Phosphoamino acid analysis on sugarcane NDPK1 labeled in vivo with ^{32}P orthophosphate followed by immunoprecipitation, SDS-PAGE and acid digestion are shown in Fig. 4. Phosphoserine was the only acid-stable phosphorylated amino acid in both control and HS samples. Retention of ^{32}P label in serine residues was higher in the enzyme obtained from cells incubated for 2 h at 25°C as compared to the sample from cells incubated at 40°C. Heat treatment resulted in approximately 40% reduction in labeled phosphate associated with serine residues. This result suggests a direct relationship between heat treatment and phosphoserine dephosphorylation of NDPK in sugarcane cells.

HS enhances NDPK activity in cultured sugarcane cells but not in tobacco cells

Total cellular proteins were partially purified to separate contaminating nucleoside triphosphatases, in order to prevent possible interference with the NDPK assay. Partially purified sugarcane protein extracts from 40°C HS treated cells showed more than 1.5-fold higher NDPK activity as compared to the control sugarcane cell extracts (Fig. 5). This HS-enhanced NDPK enzyme activity parallels the in vivo serine dephosphorylation of NDPK (Fig. 4) and the increased potential to autophosphorylate in gels (Fig. 2A). Since the mRNA and protein levels of NDPK did not change in response to HS, the increased NDPK activity strongly suggests that HS treatment activates the enzyme. In contrast to the sugarcane enzyme, NDPK activity in extracts from tobacco cells did not increase in response to HS (Fig. 5) also in accordance with the unchanged *in-gel*

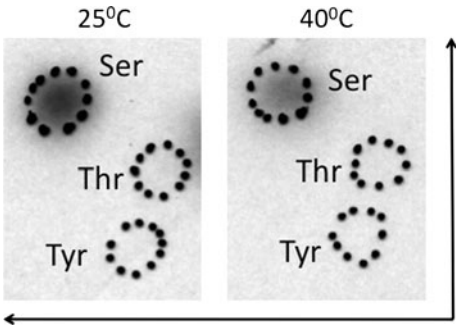

Fig. 4 Serine is the only acid-stable phosphoamino acid in NDPK. Sugarcane cells were in vivo labeled with ^{32}P-orthophosphate and subjected to treatment at 25°C (control) or 40°C (HS) for 2 h. NDPK in total proteins extracted from treated cells were immunoprecipitated with spinach NDPK I. Region corresponding to the 16.5 kDa were excised and phosphoamino acid analysis was performed as described in "Methods"

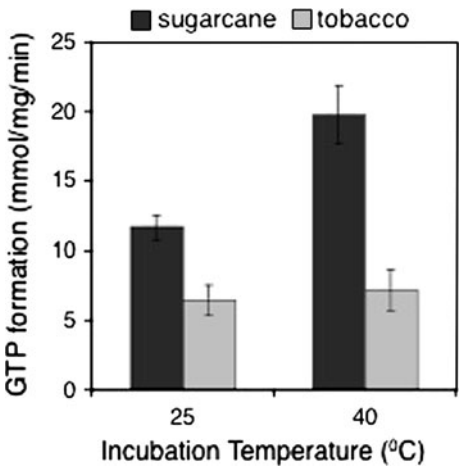

Fig. 5 NDPK activities of sugarcane and tobacco cells in response to HS at 40°C for 2 h. Enzyme activities of control (25°C) and heat-shocked (40°C) samples were determined by the transfer of labeled phosphate from γ[^{32}P]ATP to GDP to form GTP

autophosphorylation status of NDPK in tobacco cells during HS (Fig. 2b).

Discussion

The results presented in this paper indicate that sugarcane cell culture used in this experiment carry more than one isoform of NDPK1, with slightly variable isoelectric points. We have cloned two isoforms of NDPK1, analyzed its predicted protein, and characterized the behavior of NDPK1 enzyme in cells in response to heat-shock treatment.

In the predicted sugarcane NDPK1 amino acid sequence, the region corresponding to 'N^{112}-V–I-H-G-S-D-S-I' is apparently the catalytic site, with H^{115} being the phosphorylated residue in the primary phosphotransfer process, as predicted by Prosite (Expasy) analysis and based on other known NDPK sequences (Mehta and Orchard 2009). Serine phosphorylation is usually observed as a secondary process via intra- or inter-molecular phosphate transfer from the phosphohistidine intermediate. NDPK monomers from cultured sugarcane cells undergo autophosphorylation after separation by SDS-PAGE and renaturation *in-gel* (Moisyadi et al. 1994). While the exact residue that undergoes this phosphorylation is currently unknown, three phosphorylatable serine residues as predicted by Prosite domain analysis (S^{23}rfE, S^{43}faE, and S^{53}sK) may be among the potential sites of serine phosphorylation. Interestingly, of these three suggested sites of serine phosphorylation, S23 and S43, which are present in the sugarcane NDPK1 sequence, are not present in the tobacco NDPK1 sequence (Fig. 1). Thus, it can be assumed that these sites contribute to the different patterns of autophosphorylation observed in sugarcane and tobacco. Which of these serines are the primary sites of phosphorylation is currently not known. Further analysis of the sugarcane NDPK1 sequence by deleting each candidate serine residue followed by phosphorylation may provide an answer to this question.

Due to the small molecular mass of NDPK1, incorporation of Histone H1 type IIIS in the SDS gel matrix was necessary to reduce the leakage of NDPK protein from the gel during prolonged incubation and washing steps. When the 16.5 kDa [γ-^{32}P] ATP phosphorylated band was excised from the gel and Histone H1 was separated from NDPK1 by gel electrophoresis, the phosphorylation was detected only in NDPK1. This observation further confirmed that NDPK1 did not act as a protein kinase to phosphorylate Histone, but underwent autophosphorylation only. The role of Histone H1 type IIIS was limited to structural purposes in the gel matrix.

According to a previous study, the induction and decay of the autophosphorylation of NDPK1 parallels the induction and decay of low molecular weight HSPs and thermotolerance in cultured sugarcane cells (Moisyadi et al. 1994). Based on previous observations, we hypothesized that the observed HS induction of *in-gel* autophosphorylation of NDPK in sugarcane cell extracts may result from one or more of the following regulatory mechanisms: (1) induction of NDPK gene expression by HS, (2) increased mRNA stability or translation of NDPK during HS, (3) dephosphorylation of pre-existing NDPK phosphoprotein yielding more available in gel phosphorylation sites, or (4) increased ability to autophosphorylate new sites due to HS modulated conformational changes in the protein. The first two mechanisms involve changes in mRNA and protein levels, while the latter two mechanisms involve post-translational modifications of the protein. Therefore, we attempted to determine which of these mechanisms are active during HS in sugarcane cells.

The results shown in Fig. 2d and e strongly suggest that the NDPK1 mRNA level or protein level do not increase in response to HS treatments provided in this study. Thus, post-translational events are most likely to be responsible for the apparent changes in autophosphorylation of sugarcane NDPK1 in response to HS. The northern blot analysis further indicated that sugarcane NDPK1 (SoNDPK1) cDNA probe hybridized to a single band of 0.91 kb (Fig. 2c). However, purification and gel electrophoresis of sugarcane NDPKs resulted in two major groups of isoforms, NDPK1 and NDPK2, with molecular masses of 16.5 and 18 kDa, respectively. Due to high homology observed among NDPK isoforms in many organisms, we expected to observe multiple bands upon hybridization of SoNDPK1 probe with total sugarcane RNAs. The appearance of a single hybridizing band on northern blot may be explained by the possibility that sugarcane NDPK2 mRNA is similar

in length to NDPK1 mRNA. It is also possible that the level of NDPK2 mRNA was too low to be detected by hybridization with an NDPK1 cDNA probe under the stringency used in this experiment. NDPK2 associates with chloroplasts (Hasunama et al. 2003), and therefore it is not surprising that NDPK2 cDNAs are not highly abundant in cultured cells grown in dark.

Taken together, northern, western and immunoprecipitation analyses indicated that HS did not result in pronounced alterations in NDPK mRNA or protein levels sufficient to account for the increased *in-gel* autophosphorylation as observed in Figs. 2 and 3c. Thus, the above results strongly support the hypothesis that the potential for NDPK to undergo *in-gel* autophosphorylation changes during HS. Therefore, we attempted to determine if this was a direct consequence of protein dephosphorylation or exposure of potential phosphorylatable sites due to conformational changes in NDPK during HS.

According to the results of the present study, two possibilities can be suggested to explain the increased autophosphorylation of NDPK during HS. The HS treatment of cells may cause a loss of phosphate from certain pre-existing phosphoserine residues, resulting in an increase in the number of phosphorylatable serines. This would be reflected as increased *in-gel* autophosphorylation of NDPK in heat-shocked cells. However, the level of serine dephosphorylation of NDPK in heat-shocked cells (40% less than the control levels) may not account for all of the observed increase (200–300%) of NDPK autophosphorylation after gel electrophoresis. Several previous results indicate that serine phosphorylation occurs at a low stoichiometry compared to histidine phosphorylation (discussed in Hemmerich and Pecht 1992). This lack of stoichiometric phosphorylation may make it difficult to correlate the interconversion of the phosphorylated and dephosphorylated forms. In addition, other mechanisms may account for the increased ability of NDPK to undergo autophosphorylation during HS. One possibility is that conformational changes in NDPK may result in activation of the enzyme during HS, resulting in an increased catalytic activity towards the formation of NTPs, together with a decreased internal phosphate transfer to serine residues from catalytic site histidine.

According to a previous study on sugarcane NDPK1, proteins from both control and HS-treated sugarcane cells were autophosphorylated *in-gel* at phosphoserine residues, and HS-treated samples acquired a higher level of phosphoserine than control samples after *in-gel* phosphorylation (Moisyadi et al. 1994). The present study revealed the presence of phosphoserine residues in the native NDPK enzyme in vivo, confirming that phosphoserine is the only acid stable phosphoamino acid in sugarcane NDP kinase, both in vivo and in vitro. These results suggest that the increased *in-gel* autophosphorylation is at least partly due

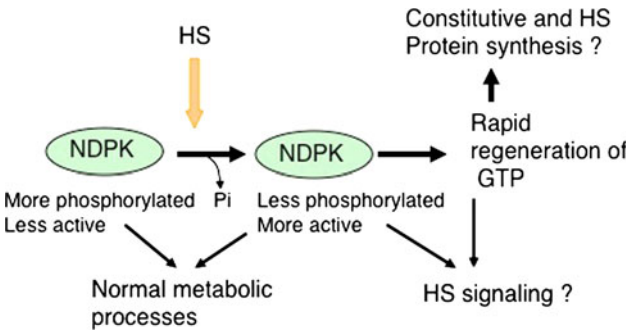

Fig. 6 A hypothetical model for HS modulation of NDPK activity, and its possible role in cellular functions during HS

to an increased in vivo dephosphorylation of the enzyme during HS. The consistent presence of phosphoserine residues further indicates that NDPK1 appears to oscillate between high and low phosphorylation states, as well as high and low enzymatic activities depending on cellular demands and environmental stimuli.

However, the increased NDPK activity and serine dephosphorylation observed during HS could also be two independent events associated with different NDPK isoforms. Studies on the precise relationship between the conformational state of the NDPK1 enzyme and its catalytic activity should provide an insight into the behavior and function of this enzyme during environmental stresses. HS induced dephosphorylation and the associated increase in NDPK activity may play a role in responding to the HS signal and the rescue of protein synthesis during HS in cultured sugarcane cells (Fig. 6), even though this is a highly speculative working hypothesis at this point. The primary role of NDPK1 enzyme has been the generation of non-adenine nucleotides and deoxynucleotides, that play essential roles in nucleic acid biosynthesis. The generation of GTP is crucial for protein biosynthesis, as elongation factors are energized essentially by GTP (Mukhopadhyay et al. 1997). Increased NDPK1 activity in sugarcane may represent continuous supply of energy source for amino acid chain elongation for protein synthesis during HS. On the other hand, cellular signaling via G-protein depends on G-protein activation by GTP binding. Thus, regeneration of GTP during HS could maximize the efficiency of HS response in sugarcane cells. Future investigations in this line may be focused on the study of direct relationship between NDPK activity, serine dephosphorylation and protein synthesis during HS.

Conclusion

The results presented in this paper indicate that sugarcane NDPK undergoes post-translational modifications in

response to HS, and these changes are the most likely contributors to the enhanced autophosphorylation shown here and in previous studies. This conclusion is strongly supported by metabolic labeling of cells with inorganic phosphate, which indicated that the actual serine phosphorylation status of NDPK changed in response to HS. In addition, such serine dephosphorylation was accompanied by an increase in enzyme activity during HS, suggesting that sugarcane cells may have a mechanism to modulate NDPK activity by post-translational modifications during HS. In contrast, NDPK in cultured tobacco cells did not show enhanced *in-gel* phosphorylation or increased NDPK activity during HS, indicating that it is differentially regulated in organisms that have different responses to HS.

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