MOLECULAR CLONING AND CHARACTERIZATION OF NUCLEOSIDE DIPHOSPHATE KINASE IN CULTURED SUGARCANE CELLS

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ABSTRACT

A low molecular weight autophosphorylating protein (pp18) in cultured sugarcane cells was identified and characterized as nucleoside diphosphate (NDP) kinase. The purified NDP kinase separated into five isoforms of 16.5kD (NDP kinase I) and two isoforms of 18kD (NDP kinase II) on two dimensional IEF/SDS-PAGE. The apparent K_m values of the purified enzyme containing both size classes were 2.3mM and 0.2mM for ATP and GDP, respectively.

Twenty one positive clones were isolated by screening a λgt11 cDNA library derived from cultured sugarcane cells with spinach NDP kinase I cDNA probe, and four were sequenced. The cDNA pSCNDK8 contained a 447 bp coding region (149 amino acids), and 58 bp and 238 bp 5' and 3' flanking sequences respectively. This cDNA hybridized to a 0.91kb mRNA. The deduced amino acid sequence of sugarcane NDP kinase cDNA clone showed over 60% sequence identity to many eukaryotic NDP kinases. The cDNA pSCNDK5 showed a frame shift resulting in 55 amino acid residues at the carboxyl terminus with no homology to NDP kinases.

The sugarcane NDP kinase showed approximately a 3 to 4-fold enhancement in *in situ* autophosphorylation and 1.5 to 2 fold increase in NDP kinase activity in response to HS at 40-42°C for 2h. However, NDP kinase protein or mRNA levels did not show an increase during HS. The synthesis and phosphorylation of NDP kinase appears to be developmentally and heat shock regulated. NDP kinase levels were highest and showed a greater enhancement in autophosphorylation in response to HS in cells in fresh culture. In contrast to cultured sugarcane cells, mRNAs for NDP kinase I enhanced at least 2-fold in response to a 2 h HS at 40°C in young sugarcane shoots.

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LIST OF ABBREVIATIONS

A	ampere
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CaM	calmodulin
cAMP	cyclic AMP
CAPS	(3-[cyclohexylamino]-1-propanesulfonic acid
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopronyl)-dimethylammonio]-1-propanesulfonate
cm	centimeter
CNBr	cyanogen bromide
cpm	counts per minute
DEAE	diethylamine
DG	diacylglycerol
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
g	gram
g	gravity
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
h	hours
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HS	heat shock
HSC	heat shock cognate
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
IEF	isoelectric focusing
IP_3	inositol 1,4,5-triphosphate
kb	kilobase
kD	kilodalton
LSB	Laemmli's sample buffer

M	molar
MES	2-(N-morpholino)ethanesulfonic acid
min	minutes
m	milli-
μ	micro-
mRNA	messenger RNA
n	nano-
NBT	nitroblue tetrazolium
NDP	nucleoside diphosphate
NP-40	nonidet P-40
PAGE	poly acrylamide gel electrophoresis
PEI	polyethyleneimine
PI	phosphatidylinositol
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol bisphosphate
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
poly(A)	poly adenylated
PVDF	polyvinyl difluoride
RNA	ribonucleic acid
s	seconds
SDS	sodium dodecyl sulfate
tRNA	transfer RNA
TRIS	tris(hydroxymethyl)amminomethane
v	volts

CHAPTER I

LITERATURE REVIEW

NUCLEOSIDE DIPHOSPHATE KINASE

INTRODUCTION. Nucleoside diphosphate (NDP) kinase (EC 2.7.4.6) is a ubiquitous enzyme which catalyzes the reversible transfer of the γ-phosphate group from a nucleoside triphosphate to a nucleoside diphosphate via a ping-pong mechanism involving a high energy phosphorylated enzyme intermediate:

$$N_1TP + E = N_1DP + E\text{-}P$$

$$E\text{-}P + N_2DP = N_2TP + E$$

in which, N_1 and N_2 may be any ribonucleoside or deoxyribonucleoside, respectively (Parks and Agarwal, 1973). The enzyme shows a broad specificity for different NTPs and NDPs as phosphate donors and acceptors (Mourad and Parks, 1966; Robinson et al., 1981; Munoz-Dorado et al., 1990; Jong and Ma, 1991). This enzyme is at the terminal step in generating all NTPs and dNTPs other than ATP thus providing a link between oxidative phosphorylation and nonadenosine NDPs (Ingraham and Ginther, 1974). It was first described in 1953 (Krebs and Hems, 1953; Berg and Joklik, 1953), and since being

investigated at biochemical, structural, regulatory and molecular levels in various organisms including both eukaryotes and prokaryotes.

STRUCTURE AND REGULATION OF NDP KINASE. The subunit molecular mass of NDP kinase ranges from approximately 16kD to 19kD, and the native enzyme exists as a trimer, tetramer or hexamer, depending on the organism (Nickerson and Wells, 1984; Munoz-Dorado et al., 1990; Nomura et al., 1991; Jong and Ma, 1991; Hemmerich and Pecht, 1992; Dumas et al., 1992; Williums et al., 1993). However, the quaternary structure is not essential for the NDP kinase activity (Lascu et al., 1992, Williums et al., 1993). At least two isoforms of NDP kinase are present in higher eukaryotes including rat (Shimada et al., 1993), mouse (Urano et al., 1992), human (Stahl et al., 1991) and spinach (Nomura et al., 1991) suggesting that it may exist as both homooligomeric as well as heterooligomeric complexes. In humans, two isoforms (Nm23 H1 and H2) are highly identical at the amino acid level (89% identical) but show different isoelectric properties (Gilles et al., 1991) and differentially distributed in cell types (Stahl et al., 1991). In rats, two isoforms (NDP kinase α and β) are differentially expressed in various tissues (Ishikawa et al., 1992). Spinach NDP kinase I and II isoforms are encoded by separate genes and show different kinetic properties (Nomura et al., 1991). These

observations suggest that different isoforms of NDP kinase may possess independent roles in the cell.

The NDP kinase function of the enzyme is mediated by phosphorylation of a histidine residue in the presence of NTP and donating the phosphate group onto an NDP. This histidine phosphorylation is characterized by its acid-labile nature (Postel and Ferrone, 1994 and references therein). The autophosphorylation on histidine shows a very rapid turnover (Munoz-Dorado et al., 1993). Substitution of the histidine^{118} with glycine in Nm23-1 (MacDonald et al., 1993) or histidine^{122} with a phenylalanine in Nm23-2 abolishes the NDP kinase activity (Postel and Ferrone, 1994). In an independent study, Hemmerich and Pecht (1992) suggest an aspartyl or glutamyl residue as the autophosphorylated residue in NDP kinase reaction in rat mucosal mast cells.

Hydrolysis of NTP and transfer of phosphate group onto the histidine residue, as well as subsequent phosphorylation of NDP are catalyzed by Mg^{2+} ions (Sedmak and Ramaley, 1971; Williums et al., 1993), however, in *Myxococcus xanthus* NDP kinase, the formation of a phosphoenzyme intermediate can occur in the absence of divalent cations (Munoz-Dorado et al., 1990). The requirement of Mg^{2+} could be fulfilled by Mn^{2+} ions in *Bacillus subtillis* NDP kinase (Sedmak and Ramaley, 1971).

In addition to the acid-labile phosphohistidine residue, serine phosphorylation of NDP kinase often occurs at a lower stoichiometry (Hemmerich and Pecht, 1992; Munoz-Dorado et al., 1993; Engel et al., 1994; Bominaar et al., 1994). This serine phosphorylation is often suggested to occur as an intra- or inter-molecular phosphate transfer from phosphohistidine enzyme intermediate of NDP kinase (Munoz-Dorado et al., 1993). *In vitro*, casein kinase is also capable of phosphorylating serine residues on Nm23-1, however, the physiological significance of this is not clear (Engel et al., 1994). Interestingly, in recombinant Nm23-1 in which the critical histidine residue is substituted with a glycine residue which abolishes NDP kinase activity, serine phosphorylation is not affected (MacDonald et al., 1993). Additionally, serine phosphorylation in recombinant and *in vivo* labelled human Nm23-1 far exceeds the histidine phosphorylation (MacDonald et al., 1993) in contrast to low stoichiometry observed in other NDP kinases, suggesting a more important role for serine phosphorylation in Nm23-1. However, a recent report indicated that active site histidine was essential for autophosphorylation of non-histidine residues (Bominaar et al., 1994). Bominaar et al. (1994) also suggest that the observations made by MacDonalds et al. (1993) that serine autophosphorylation was independent of active site histidine phosphorylation may be due to contamination of their non-functional mutant enzyme preparations with functional NDP kinase.

The X-ray crystallographic structures of NDP kinase are available for the purified proteins from *Dictyostelium discoideum* and *Myxococcus xanthus* (Dumas et al., 1992; Williams et al., 1993; Morera et al., 1994). In the *Dictyostelium* NDP kinase, crystal structure is in perfect agreement with the proposed His^{122} as the phosphoamino acid residue (Williams et al., 1994). In both *Dictyostelium* and *Myxococcus* enzymes, the binding of nucleotide was through the sugar and phosphate groups, with the base directed outside explaining the broad specificity of NDP kinases toward the nucleotide substrate (Morera et al., 1993; Williams et al., 1994).

FUNCTIONS OF NDP KINASE. In contrast to the initial belief that NDP kinase is a housekeeping enzyme that maintains NTP and dNTP levels in cells at the expense of ATP (Krebs and Hems, 1953), recent experiments have attributed several important functions for this enzyme. The abundance and distribution of this enzyme in different tissues support this notion of multiple functions. For example, in red blood cells, the activity of NDP kinase is about 10-fold higher than that of glycolytic enzymes (Parks and Agarwal, 1973) and in bovine brain, NDP kinase constitutes between 0.1-0.2% of total proteins (Nickerson and Wells, 1984). Nucleoside diphosphate kinases may play a significant role in plasma membrane G-protein regulated processes either directly or indirectly by maintaining available GTP levels (Randazzo et al.,

1991; 1992). It exists in association with various other proteins that require GTP as a factor, such as small G-proteins (Teng et al 1991; Ruggieri & McCormick, 1991), protein synthesis initiation factor eIF2 (Walton & Gil, 1975) and microtubules (Nickerson & Wells, 1984). Membrane associated NDP kinases may play a role in the regulation of adenylate cyclase (the enzyme involved in the generation of cyclic AMP) by channeling GTP onto the GTP binding protein G_s, in a hormone dependent manner (Kimura and Shimada, 1986). It has also been suggested a regulatory role in DNA and RNA syntheses by providing dNTPs and NTPs, respectively (Testa & Benerjee, 1979), and in the intracellular energy metabolism (Kavanaugh-Black et al., 1994).

The recent discovery that the proteins controlling tumor metastasis in mammals (Nm23 H1) (Steeg et al, 1988) and normal development in *Drosophila* (Awd) (Biggs et al, 1990) are NDP kinase homologs (Rosengard et al., 1989) attracted an added importance to this enzyme. Reduced transcript levels of nm23-1 is associated with the high metastatic potential of human breast carcinomas (Bevilacqua et al., 1989). One of the suppressor proteins for differentiation of mouse leukemic cells has been identified as Nm 23/NDP kinase (Okabe-Kado et al., 1992). Interestingly, serine phosphorylation, and not NDP kinase activity of human Nm23 H1 correlates with suppression of metastatic potential of tumor cells (MacDonald et al., 1993). In contrast to the

above findings, the over-expression of *nm23* mRNA is associated with spontaneous metastasis in rat lung osteosarcomas (Honoki et al., 1993). Increased NDP kinase activity has also been detected in malignant colon cells compared to normal cells (Francis et al., 1989), suggesting a variable role for Nm23 in different types of tumor cells. Human Nm23-H2, which also shows NDP kinase activity, functions *in vitro* as a transcription factor (PuF) for *c-myc* gene (Postel et al., 1993). Destruction of NDP kinase activity by a point mutation that disrupts the catalytic site involved in phosphate transfer does not affect its ability to act as an *in vitro* transcription factor (Postel and Ferrone, 1994). These observations indicate that NDP kinase proteins are multi-functional molecules with important regulatory roles other than generating NTPs in cells.

HEAT SHOCK RESPONSE

All living organisms respond to moderate increases in temperature by altering the normal cellular functions. This phenomenon, commonly called the heat shock response (HSR) is one of the most extensively studied aspects in science since its discovery in *Drosophila* about three decades ago (Ritossa, 1962; 1964). In recent years, HSR in plants, animals and prokaryotes have been the subject of intense investigation and reviewed thoroughly (Burdon,

1986; Lindquist, 1986; Lindquist and Craig, 1988; Vierling, 1992; Feige and Mollenhauer, 1992).

One component of HSR is the induction or dramatic enhancement of the synthesis of a set of proteins designated as heat shock proteins (HSPs) (Tissieres et al., 1974). The induction of HSPs in response to heat stress is shown in various systems including plants, animals and bacteria (Ashburner and Bonner, 1979; Barnett et al., 1980; Key et al., 1981; Yamamori and Yura, 1982; Cooper and Ho, 1983; Moisyadi and Harrington, 1989) with only a few exceptions (Dura, 1981; Wittig et al., 1983; Xiao and Mascarenhas, 1985;). However, the minimum temperature required to induce a full complement of HSPs is different in various species (Key et al., 1981; Cooper and Ho, 1983; Daniels et al., 1985; Somers et al., 1989).

Another component of the HSR is the marked reduction of the synthesis of most of the normally expressed proteins (Kelley and Schlesinger, 1978; Key et al., 1981; Lindquist, 1981). This can be a direct consequence of the inhibition of normal gene transcription (Spradling et al., 1975; Jamrich et al., 1977; Findly and Pederson, 1981) and the inhibition of pre-existing message transcription (Lindquist, 1980). Reduced translation of normal messages could also be due to altered mRNA processing (Neumann et al., 1984; Yost and

Lindquist, 1986), mRNA stability (Lindquist, 1980) or modifications in ribosomes (Scharf and Nover, 1982). Interestingly, in many tropical crops including maize and sugarcane, heat shock does not result in marked supression of normal protein synthesis (Cooper and Ho, 1983; Mansfield and Key, 1987: Moisyadi and Harrington, 1989) however, the molecular aspects underlying this behavior is still unknown.

Induction of heat shock proteins at sublethal temperatures is usually accompanied by an acquisition of thermotolerance to otherwise lethal temperatures (McAlister and Finkelstein, 1980; Lin et al., 1984; Harrington and Alm, 1988; Moisyadi and Harrington, 1989). It has been suggested that at least some HSPs play a role in acquisition of thermotolerance (Li and Laszlo, 1985; Sanchez and Lindquist, 1990). Despite the magnitude of work done on the induction of HSPs and thermotolerance, there is no direct evidence to show their precise roles in development of thermotolerance.

Several structural rearrangements have been reported in cells subjected to heat shock. These include rapid ultrastructural changes in the nucleolus (Fransolet et al., 1979; Neumann et al., 1984; Pelham, 1984; Welch and Suhan, 1985) and assembly of cytoplasmic aggregates known as heat shock granules (Arrigo and Ahmad-Zadeh, 1981; Neumann et al, 1984). Other

effects of heat shock are the changes in post-translational modifications of proteins such as ubiquitination (Levinger and Varshavsky, 1982; Shimogawara and Muto, 1989), phosphorylation and dephosphorylation (Glover, 1982), acetylation and methylation (Arrigo, 1983).

HEAT SHOCK PROTEINS AND THEIR FUNCTIONS. Heat shock proteins can be divided into several classes based on their molecular weights as HSP110. HSP90, HSP70, HSP60 and low molecular weight HSPs of 15-30kD (Vierling, 1991). In eukaryotes, HSPs are encoded by multi-gene families, and consist of several isoforms belonging to each size class (Lindquist and Craig, 1988). In animals, the high molecular weight proteins constitute the major group of HSPs; while in plants, low molecular weight HSPs are produced as the major group (Mansfield and Key, 1987; Hornandez and Vierling, 1993). Cytoplasmic and organelle homologs to many of these HSPs, particularly the high molecular weight proteins, synthesized constitutively under non-stress conditions are called heat shock cognate proteins (HSCs) (Ingolia and Craig, 1982a; Pelham, 1986).

Several recent reviews discuss in detail the occurrence and possible involvement of HSPs in both eukaryotic and prokaryotic HSR (Lindquist, 1986; Lindquist and Craig, 1988; Pelham, 1990; Vierling, 1991). The striking

homology between high molecular weight HSPs and their HSC counterparts raise the possibility that HSPs carry out similar functions during heat shock. All HSP70 homologs bind ATP and show weak ATPase activity (Flaherty et al., 1990; Pelham, 1990). It has been suggested that HSP70 homologs (including HSC70s) play a role in ATP-dependent protein folding, unfolding and assembly during normal growth and heat shock (Chirico et al., 1988). Transport of proteins across ER and mitochondrial membranes is facilitated by HSP70 homologs in yeast (Chirico et al., 1988). High molecular weight HSPs especially HSP60s function as "molecular chaperons", the molecules involved in mediating the translocation, as well as folding and assembly of other proteins (Ellis and Hemmingsen, 1989; Martin et al., 1992).

Analyses of genes encoding low molecular weight HSPs in plants indicate that most of them belong to four multi-gene families (Vierling, 1991). Despite the abundant synthesis of low molecular weight HSPs in plants, their possible functions in the HSR is still not clear. Their contribution to formation of HS granules which contain large amounts of normal mRNA suggest that they may be preserving normal mRNAs during heat shock (Nover et al., 1989). High homology of some low molecular weight HSPs to α-crystallin in animals (Ingolia and Craig, 1982b; Bossier et al., 1989) raise the possibility of a structural role in maintaining cellular integrity during heat shock (Lindquist and

Craig, 1988). The presence of low molecular weight HSPs in various cellular compartments also suggests a possible chaperonin-like function (Vierling, 1991).

STRUCTURE AND TRANSCRIPTIONAL REGULATION OF HEAT SHOCK GENES. A general characteristic of heat shock induced or enhanced genes in eukaryotes is the presence of highly conserved sequence motif called as heat shock element (HSE) 5' to the TATA box in the promoter region (Pelham, 1982). Heat shock elements are inverted repeats of the sequence NGAAN, usually represented by more than one sequence per gene (Dudler and Travers, 1984; Gurley et al., 1986). Heat shock transcription factors (HSF) that bind to HSE has been isolated and characterized from several organisms (Parker and Topol, 1984a,b; Wiederrecht et al., 1987; Nieto-Sotelo et al., 1990).

Two mechanisms have been suggested for HSE-HSF regulation of heat shock gene expression. In yeast, HSF is constitutively bound to HSE, and is activated by phosphorylation upon heat shock conditions resulting in induced heat shock gene expression (Sorger et al., 1987; Sorger and Pelham, 1988; Nieto-Sotelo et al., 1990). In *Drosophila*, binding of HSF with HSE is induced by phosphorylation of the HSF upon heat shock. Moreover, in *Drosophila* heat

shock mRNA are transcriptionally initiated but arrested after partial elongation. The binding of activated HSF with HSE facilitates the elongation of HS mRNA (Gilmour and Lis, 1986; Rougvie and Lis, 1988).

TRANSLATIONAL REGULATION. Translational regulation of protein synthesis during heat shock has been studied mostly in *Drosophila* (Lindquist, 1980, 1981; Scott and Pardue, 1981; Sanders et al., 1986). During first few minutes of heat shock, translation of normal messages is dramatically inhibited and polysomes are dissociated. When heat shock mRNAs appear in the cytoplasm, they are translated at a very high efficiency in newly assembled heat shock polysomes (Lindquist et al., 1975). The molecular mechanisms of preferential translation of heat shock messages over constitutive messages are still largely unknown. Scott and Pardue (1981) suggest that ribosomes determine the translational preference in *Drosophila* heat shock mRNAs, however, a role of soluble cytoplasmic factors has been suggested (Sanders et al., 1986). Although the soluble factors responsible for rescuing normal protein synthesis are still uncertain, many studies point at the role of translation initiation factors.

Initiation of protein synthesis has several steps catalyzed by a number of initiation factors (Hunt, 1980; Pain, 1986). In eukaryotes, initiation factor 2

(eIF2) catalyzes the critical binding step of the special initiator tRNA (Met-$tRNA_i$, carrying the methionine group) to the small subunit (40S) of ribosomes. The eIF2 factor is comprised of 3 subunits α, β and θ. The eIF2α subunit binds GTP, while eIF2θ subunit binds Met-$tRNA_i$ (Lloyd et al., 1980). Subsequent formation of translationally active 40S/60S ribosomal complex results in hydrolysis of GTP to GDP (Jagus et al., 1981).

Formation of active initiation complex is dependent on GDP:GTP ratio, where cytoplasmic GTP level should exceed GDP level by an order of magnitude (Walton and Gil, 1972). High levels of GDP are inhibitory for both eukaryotic and prokaryotic initiation complexes (Walton and Gil, 1972; Hamel and Cashel, 1974). Thus, constant removal of GDP is necessary to maintain the initiation of protein synthesis. Nucleoside diphosphate kinase may play a role in regenerating GTP from GDP, thereby lowering the GDP:GTP ratio in the vicinity of protein synthetic machinary (Walton and Gil, 1972). Supporting this idea, association of NDP kinase with eIF2 has been demonstrated in rat liver reticulocytes (Walton and Gil, 1972).

Heat shock induced phosphorylation of eIF2α is shown in eukaryotic cells (Ernst et al., 1982; Duncan and Hershey, 1984). Phosphorylated form of eIF2α prevents the formation of active eIF2α.GTP complex inhibiting the

initiation of protein synthesis (Duncan and Hershey, 1984). Heat shock induces a protein kinase (eIF2α kinase) responsible for phosphorylation of eIF2α. During heat shock, HSP90 undergoes autophosphorylation (Rose et al., 1989; Csermely and Kahn, 1991), which may play a role in stimulating eIF2α kinase activity (Rose et al., 1987, 1989; Matts et al., 1992).

PROTEIN PHOSPHORYLATION AND PROTEIN KINASES

Phosphorylation of proteins is a common cellular mechanism for modulating various physiological functions such as cell division, membrane transport of ions and metabolites, metabolic activities and gene expression (reviewed by Ranjeva and Boudet, 1987; Edelman and Krebs, 1987). Protein kinases transfer the γ-phosphate group of nucleoside triphosphate onto specific amino acid groups of the target protein. Depending on the phosphorylated amino acid, protein kinases are grouped as serine/threonine kinases and tyrosine kinases (Hunter and Cooper, 1985; Hunter, 1987; Hanks et al., 1988). Specific phosphatases dephosphorylate the phosphorylated residues on the target proteins (Cohen, 1985).

Activities of many protein kinases are dependent on or stimulated by various subcellular factors especially the second messengers involved in signal

transduction processes, including cyclic AMP, phospholipids and calcium (Soderling, 1990). Cyclic nucleotide-dependent protein kinases have been characterized thoroughly in animals and yeast (Corbin et al., 1978; Taylor, 1989; Knighton et al., 1991). Calcium-phospholipid-dependent protein kinases (protein kinase C family) are characteristic to the animal systems (NIshizuka, 1988). Calcium/calmodulin dependent protein kinases (CaM kinases) are well characterized in animal systems (Soderling, 1990) and yeast (Pausch et al., 1991). Growing evidence indicate the presence of calcium-dependent protein kinases (CDPK) (Hetherington and Trewavas, 1982; Polya and Micucci, 1984; Harmon et al, 1987; Battey and Venis, 1988; Bogre et al., 1988; Harper et al, 1991) and calmodulin dependent protein kinases (Paranjape et al., 1994; Patil et al., 1994) in higher plants. The role of calmodulin on stimulation of the activity of several plant protein kinases was also suggested (Polya and Micucci, 1984; Blowers and Trewavas, 1989).

Changes in the phosphorylation status of proteins during heat shock are observed in many systems (Scharf and Nover, 1982; Duncan and Hershey, 1984; Sanders et al., 1986; Schlesinger, 1986; Sorger et al., 1987; Sorger and Pelham, 1988). These rapid alterations in the pattern of phosphoproteins point to the existence of HS activated protein kinases and protein phosphatases. Functional significance of protein phosphorylation and dephosphorylation during

heat shock has been suggested in several instances. Rapid phosphorylation of animal HSP27 after onset of heat shock has been interpreted as a protective measure against heat induced cascade reactions leading to cellular malfunctioning (Landry et al., 1989). Autophosphorylation of HSP90 during heat shock is considered as a regulatory mechanism of its enzymatic action (Rose et al., 1987; Csermely and Kahn, 1991).

SIGNAL TRANSDUCTION

The transmission of extracellular signals across the plasma membrane into the target units via second messengers such as inositol 1,4,5 triphosphate (IP_3), diacylglycerol (DG), cyclic AMP (cAMP) and Ca^{2+} is well characterized in animal systems (Nishizuka, 1986; Berridge, 1987). In the inositol triphosphate mediated process, receptor molecules on the plasma membrane activate an intrinsic plasma membrane GTP-binding protein (G-protein) upon external signal (Gilman, 1987; Lochrie and Simon, 1988; Neer and Clapham, 1988).

The G protein consists of 3 subunits α, β and γ. Upon activation, the bound GDP on inactive Gα subunit is replaced by GTP. The Gα-GTP (active form) is then able to dissociate from the other 2 subunits of the G protein complex and activate the effector molecule, phospholipase C (PLC). After

effector activation, the GTPase activity of the activated Gα hydrolyses bound GTP to GDP converting to the inactive form leading to the reassociation of G protein subunits (Gilman, 1987).

The mechanism of G-protein activation via conversion of bound GDP to GTP is not clear. Nucleoside diphosphate kinase may be responsible for direct transfer of a phosphate group from ATP to the bound GDP on the regulatory G-proteins (Ohtsuki and Yokohama, 1987; Ohtsuki et al., 1987; Kikkawa et al., 1990; Randazzo et al., 1991). However, a recent controversial report suggests that NDP kinase phosphorylates only free GDP but not the G-protein bound form and may not play a strictly regulatory role in G-protein activation (Randazzo et al., 1992).

The G-protein activated PLC hydrolyses plasma membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2) releasing the second messengers, IP_3 to the cytoplasm and DG on the membrane surface. Diacylglycerol stimulates protein kinase C in animal cells leading to a cascade of protein phosphorylations transmitting the external signal to the target molecules (reviewed by Nishizuka, 1988). Inositol triphosphate released to the cytoplasm mobilizes calcium from internal pools, activating various calcium sensitive cellular processes including protein kinase activities (Berridge and Irvine, 1989). Cooperative stimulation of two pathways involving DG and IP_3 by each

other further amplifies the efficiency of signal transduction system (Berridge, 1987).

Inositol triphosphate is converted to inositol via a progressive dephosphorylation. Alternatively, it can be converted into other higher phosphorylation states and dephosphorylated to inositol via several pathways. The exact role of many of the other inositol phosphates is not certain (Berridge and Irvine, 1989). Diacylglycerol is converted to CMP-phosphatidate and associates with inositol to form phosphatidylinositol (PI) in the membrane. This is further phosphorylated to phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) by the activities of PI kinase and PIP kinase respectively, completing the cycle (Berridge and Irvine, 1989).

Phosphoinositol metabolism is not limited to the plasma membrane. Interaction between PIP_2 and cytoskeletal actin binding proteins (ABPs) such as profilin is demonstrated in mammalian systems (Lassing and Linberg, 1985). The ABPs may play a regulatory role by interaction with PIP_2 preventing its hydrolysis by PLC, controlling the available PIP_2 for signalling process under normal conditions (Goldschmidt-Clermont et al., 1990). Subcellular localization experiments in mammalian cells show that PIP can be synthesized intracellularly before it is translocated to the plasma membrane (Lundberg and

Jergil, 1988). Several enzymes of phosphoinositol mediated signaling pathway including PI kinases, PLC and DG kinase are also associated with cytoskeleton of animal cells (Nahas et al., 1989; Grondin et al., 1991). The existence of the components of phosphoinositide cycle and lipid phosphorylation has been demonstrated in the nuclei of animal cells (Capitani et al., 1989; Divecha et al., 1991). The exact role of nuclear PI metabolism is not clear, however, the increased DG level may stimulate the translocation of PKC to the nucleus (Divecha et al., 1991).

Evidence for components of inositol triphosphate and diacylglycerol mediated signalling processes has been obtained in many plant systems (Sandelius and Sommarin, 1986; Sommarin and Sandelius, 1988; Boss and Morre, 1989; Einspahr and Thompson, 1990; Lehle, 1990; Drobak et al., 1991); however, compared to animal systems, the role and importance of this pathway in plant signal transduction is not well understood. Guanosine triphosphate-binding proteins have been identified in higher plant plasma membranes (Blum et al., 1988). Preliminary evidence for the role of G-proteins in hormone signal transduction has been demonstrated in rice (Zaina et al., 1990). Both PI kinase and PIP kinase activities are present in plasma membranes of dark grown wheat (Sommarin and Sandelius; 1988). Isolated nuclear fractions from carrot protoplasts contain PIP and PIP_2, and also show

weak PI kinase activity, although much less than the plasma membrane associated activity (Hendrix et al., 1989). Recently, the association of PI kinase with cytoskeleton is shown in carrot protoplasts (Xu et al., 1992). These findings suggest a high similarity between plant and animal PI metabolism; however, several unique features in plants are apparent.

In plant cells, ratio of PIP to PIP_2 is much higher than in animal systems. The turnover rate of PIP is extremely fast in plant cells (Drobak et al., 1988; Irvine et al., 1989) suggesting that the signalling processes in plants and animals may use similar components in different manners. The presence of cAMP has been shown in plants, however, its involvement in signal transduction pathways has not been detected (Renelt et al., 1993).

Rapid changes in phospholipid metabolism, intracellular calcium levels and protein phosphorylation have been shown in plant tissues exposed to environmental signals. In *Samanea saman* pulvini, a brief pulse of white light stimulates increases in IP_3 and DG, and decreases in PIP and PIP_2 within seconds (Morse et al., 1987, 1989). A thirty second hyperosmotic shock increases plasma membrane DG level by 40% in *Dunaliella salina* (Ha and Thompson, 1991). A similar rapid response in PIP_2 turnover occurs in response to auxin stimulus in *Catheranthus roseus* (Ettlinger and Lehle, 1988).

Plant cells release intracellular Ca^{2+} when treated with IP_3 (Reddy and Pooviah, 1987; Schumaker and Sze, 1987; Gilroy et al., 1990) suggesting the presence of endogenous IP_3 mediated calcium releasing system in plant cells.

Interactions between second messengers and protein phosphorylation has been suggested as a major regulatory mechanism in plant responses to external signals (Blowers and Trewavas, 1989). Further supporting this idea are rapid changes in calcium-dependent protein phosphorylation within minutes in response to elicitor signal in cultured tomato cells (Felix et al., 1991).

Heat shock stimulates turnover of phosphoinositides, and increases IP_3 and intracellular calcium levels in animal cells (Stevenson et al., 1986; Calderwood et al, 1987, 1988). Presently there are no reports for the heat shock induced changes in components of the IP_3 pathway in plant systems, however, preliminary work on cultured sugarcane cells in response to temperature shows rapid changes in phosphoinositide metabolism including a marked increase in IP_2 levels within 1 min after 36°C HS (Harrington et al., unpublished data). Existence of all the components of inositol phospholipid-mediated signalling pathway in plants suggest that heat shock signal transduction may be controlled by a process similar to that in animals.

CHAPTER II

SIGNIFICANCE AND HYPOTHESES

Heat shock response in cultured sugarcane cells is rather unique in that the normal protein synthesis is not suppressed during a sublethal heat shock which induces a full complement of HSPs (Moisyadi and Harrington, 1989). A similar phenomenon is observed is maize which is also a related C_4 plant (Cooper and Ho, 1983; 1984). Functional analysis of proteins induced or enhanced during HS in these species may provide important insights into the unique features of cellular metabolism, especially protein synthesis under stress conditions.

Earlier work carried out in this laboratory has focused on the characterization of a low molecular weight protein in cultured sugarcane cells. This protein showes an enhanced autophosphorylation activity when cells are subjected to heat shock at 36°C (Harrington et al., 1990). The kinetics of induction and decay of the activity of 18kD protein parallels the induction and decay of low molecular weight HSP synthesis and acquired thermotolerance (Moisyadi and Harrington, 1989). This protein autophosphorylates exclusively on serine residues, and the phosphate group of the autophosphorylated protein

shows very rapid turnover. The purified protein phosphorylates histone H1 *in situ* on gels or *in vitro* when supplied with γ-^{32}P-ATP (Moisyadi, 1991)

Further characterization of this autophosphorylating protein from sugarcane reveals similarity to an 18kD autophosphorylating protein designated as pp18, located in pea shoot plasma membrane fractions and characterized as a catalytic subunit of a protein kinase (Hetherington and Trewavas, 1982; 1984; Blowers et al., 1985). The phosphate group of the autophosphorylated protein shows very rapid cycling as demonstrated by pulse chase experiments (Blowers and Trewavas, 1989), and the protein kinase activity of this protein is stimulated by calcium and calmodulin (Hetherington and Trewavas, 1982;1984; Blowers and Trewavas, 1987). In addition, purified pp18 from pea plasma membrane shows phosphatidylinositol kinase (PI kinase) activity, phosphorylating PI to form phosphatidylinositol phosphate (PIP) (Blowers and Trewavas, 1988). Based on the plasma membrane localization and preliminary biochemical characteristics of pp18, Blowers and Trewavas (1988; 1989) suggest a role in signal transduction processes.

To characterize pp18 in sugarcane at biochemical and molecular level, the protein was purified and sequenced. A 13 amino acid internal fragment of the affinity purified pp18 from sugarcane showed a very high homology to a

conserved region of nucleoside diphosphate (NDP) kinase from spinach and several other sources (Kimura et al., 1990; Lacombe et al., 1990; Nomura et al., 1991).

Nucleoside diphosphate kinases have been purified and characterized from various animal and bacterial sources (Nickerson and Wells, 1984; Kimura and Shimada, 1988; Prescan et al., 1989; Munoz-Dorado et al., 1990), yeast (Jong and Ma, 1991) and spinach (Nomura et al., 1991). The genes or cDNAs encoding NDP kinase from animals (Ishikawa et al., 1992; Shimada et al., 1993), bacteria (Munoz-Dorado et al., 1990; Hama et al.,1991), slime molds (Lacombe et al., 1990) and several higher plants (Nomura et al., 1992; Yano et al., 1993; Zhang et al., 1993; Finan et al., 1994; Harris et al., 1994) have been cloned and sequenced. There are no reports on the characteristics of this important protein and its cDNA sequence from a tropical C_4 plant such as sugarcane in which normal protein synthesis is maintained during heat shock. An analysis of the regulation of pp18 synthesis, phosphorylation and enzymic activity during heat shock, as well as deducing the primary structure of pp18 protein should facilitate our understanding on how tropical crops respond to increased temperature.

HYPOTHESES

Based on the existing knowledge on the low molecular weight phosphoprotein from sugarcane and pea, the present research project was planned to test the following hypotheses.

1) The autophosphorylating low molecular weight protein (pp18) in sugarcane is a nucleoside diphosphate kinase.

2) The increased autophosphorylation of pp18 during heat shock is due to increased synthesis.

The above hypotheses will be tested by the following major approaches;

a) Purification of pp18 from cultured sugarcane cells, and identification and characterization of its enzymic activity.

b) Cloning and sequencing cDNAs that encode possible isoforms of pp18.

c) Analysing the changes in mRNA levels, protein levels and kinase activity of pp18 in response to heat shock.

CHAPTER III

PURIFICATION OF PP18 AND CHARACTERIZATION AS NUCLEOSIDE DIPHOSPHATE KINASE

INTRODUCTION

The enzyme NDP kinase was first described more than 4 decades ago (Krebs & Hems, 1953; Berg & Joklik, 1953). This enzyme has since been purified and characterized from several organisms including animals (Kimura and Shimada, 1988; Robinson et al., 1981; Prescan et al., 1989; Buczynski and Potter, 1990), bacteria (Munoz-Dorado et al., 1990; Kavanaugh-Black et al., 1994), yeast (Jong and Ma, 1991), spinach (Nomura et al., 1991) and pea (White et al., 1993). The purified enzyme shows a subunit molecular mass of 16kD to 19kD in different organisms.

Previous work in this laboratory on heat shock response was focused on characterization of a low molecular weight phosphoprotein (pp18) in cultured sugarcane cells (Moisyadi, 1991). Attempts to purify pp18 by anion exchange chromatography on DEAE-Sephadex column and affinity chromatography on ATP-agarose followed by elution with 1M NaCl (pH4) resulted in low yields of purified protein (Moisyadi, 1991). The pp18 was later demonstrated to have the capacity to autophosphorylate to at least 2-fold higher intensity with GTP

than with ATP. This higher affinity of pp18 towards GTP/GDP was utilized in the present study to purify this protein in high yields. In this chapter, the modified purification protocol for rapidity and high yield, and characterization of the pp18 as an NDP kinase are described.

MATERIALS AND METHODS

Plant Materials. Suspension cultured sugarcane (*Saccharum officinarum* L. cv. H50- 7209) cells maintained as described previously (Moisyadi & Harrington, 1989) were used in all experiments. Heat shock was given to 14 day old cells at 38°C for 2 hours in an orbital shaking water bath.

Purification of pp18. The purification protocol described before (Moisyadi 1991) was modified for rapidity and higher yield of pp18. Heat shocked sugarcane cells (approximately 500g) ground in liquid N_2 were washed with 10 volumes of 100% acetone at 0°C for 2 hours, and extracted with 2 volumes of buffer A (10mM Tris pH8, 2mM EDTA, 2mM EGTA, 1%β-mercaptoethanol, 0.1% CHAPS, 1mM PMSF) by stirring for 5 hours at 0°C. The homogenate was filtered through miracloth and centrifuged at 14500g for 20 min at 1°C. Supernatant was loaded directly onto DEAE sepharose (Sigma) column (2.5 X 25cm) at 0.42ml/min rate. The column was washed with 200 mL of buffer B (10mM Tris pH8, 1mM EDTA, 1mM β-mercaptoethanol, 1mM PMSF)

containing 50 mM NaCl and proteins eluted with 0.05-0.8M NaCl gradient in buffer B. The fractions with 18kD autophosphorylating activity were pooled. The pooled peak fractions were diluted 5-fold with milli-Q H_2O adjusting the final concentrations of the components to 0.3mM sorbitol, 6mM Tris (pH7.2), 6mM MES, 0.2mM EDTA, 10 mM $MgCl_2$ and 0.1mM DTT (buffer C). This sample was loaded directly onto an ATP-agarose (Sigma) column (5mL) at 0.1mL/min rate and the column was washed sequentially by gravity, with 10 column volumes each of buffer C containing 0.3M NaCl, 20mM EDTA (without $MgCl_2$), 1M NaCl (pH9) and 1M NaCl (pH4). The pp18 was eluted by developing the column with 10 column volumes of 0.5mM GDP. The eluate was exhaustively dialyzed against 10mM ammonium bicarbonate, lyophilized and resuspended in milli-Q H_2O for further studies.

Gel electrophoresis and *in situ* phosphorylation. Protein samples from different steps of purification were separated by single-dimension SDS-PAGE as described (Moisyadi & Harrington, 1989) on minigels containing 100μg/mL histone H1 type IIIS. The gels were phosphorylated using γ-^{32}P-ATP (New England Nuclear) according to the procedure described before (phosphorylation method B, Moisyadi et al., 1994). Affinity purified proteins were also separated by single-dimension SDS-PAGE or two-dimensional IEF/SDS-PAGE on minigels as described (Moisyadi & Harrington, 1989) and silver stained.

Sequence determination. The purified protein (2 μg) was transferred onto ProBlot PVDF membrane (Applied Biosystems) after single dimension SDS-PAGE. The transfer was carried out in a buffer containing 0.1M CAPS buffer (pH11) and 10 % methanol at room temperature for 1 hour at 50V (180mA). The band corresponding to 16.5 kD region was excised and subjected to automated Edman degradation on a protein sequencer (Applied Biosystems 475A) by Dr. Thomas J. Lukas at the Department of Pharmacology, Vanderbilt University. No sequential release of phenylthiohydantoin amino acids was observed on two different preparations of the protein. The blotted protein was then subjected to cleavage by CNBr (10mg mL^{-1}) in 70% formic acid for 20 h at room temperature. The cleavage solution was removed and the membranes were then subjected to automated Edman degradation as described above. The CNBr cleavage procedure was repeated on three different samples so that amino acid sequence data could be compared and possible ambiguities resolved.

Nucleoside diphosphate kinase activity. The NDP kinase assay was performed according to a method described by Munoz-Dorado et al. (1990) with several modifications. The assay mixture (10μl total) contained 50mM HEPES (pH7.4), 100mM NaCl, 10mM $MgCl_2$, 2mM ATP, 2mM GDP, 5μg BSA and 10μCi γ-^{32}P-ATP (3000Ci/mmole). Purified enzyme (10ng/μl) was added to start the reaction and incubated at 32°C for 10 min unless specified. The

reaction was stopped by adding 5 volumes of 10mM EDTA (pH3.65). Aliquots (1μl) were loaded onto polyethyleneimine (PEI)-cellulose thin layer chromatography plates (Sigma) and separated by ascending chromatography with 0.75M KH_2PO_4 (pH3.65). The plate was dried and autoradiographed on preflashed X-ray film for 30 min. The radioactive spots corresponding to GTP were removed and quantified in a Beckman liquid scintillation counter, with 3mL of ScintiVerse E fluid (Fisher Chemical Co.).

Time course of NDP kinase reaction. The NDP kinase assay was carried out as described above except that the volume of the reaction mixture was 20μl. After incubation for the times 0, 8 sec, 15 sec, 30 sec, 1 min, 2 min, 5 min, 10 min, 30 min and 1 hr, aliquots were chromatographed as described above.

Effect of enzyme concentration on NDP kinase activity. The NDP kinase assay was performed with enzyme concentrations 0, 0.25, 0.50, 0.75, 1.0, 2.0 and 5.0ng μl^{-1} in 10μl reaction mixture. After incubation for 10 min, aliquots were chromatographed as described above.

Kinetics of NDP kinase reaction. To determine K_m(ATP), GDP concentration was kept constant at 2mM, and to determine K_m(GDP), ATP concentration was kept constant at 2mM in each reaction mixture containing variable amounts of

ATP and GDP respectively. The reaction was carried out for 10 min and aliquots were chromatographed as described above.

RESULTS AND DISCUSSION

Purification of pp18. Several steps of the original purification protocol (Moisyadi, 1991) were modified for rapidity and increased yield of pp18. The ammonium sulfate precipitation step which resulted in only 1.1 fold purification (Moisyadi, 1991) was eliminated completely. According to Robinson et al. (1981), ammonium sulfate precipitation results in the appearance of an inactive polymer of NDP kinase. This polymer cannot be renatured to resume enzymic activity thus reducing the specific activity of the purified enzyme (Robinson et al., 1981). Elimination of the ammonium sulfate precipitation step in the present protocol is essential for obtaining more accurate readings in subsequent enzyme assays of the purified protein.

The total sugarcane proteins extracted into buffer A were filtered, centrifuged at high speed and chromatographed directly onto a DEAE-sepharose column, eliminating the need for dialysis. The column was eluted with a NaCl gradient (50mM-0.8M), and pp18 activity was detected by *in situ* phosphorylation method B (Moisyadi et al., 1994). The pp18 peak activity was detected at conductivity 8.9 to 18.8 mS cm^{-1}. At this step, pp18 activity was

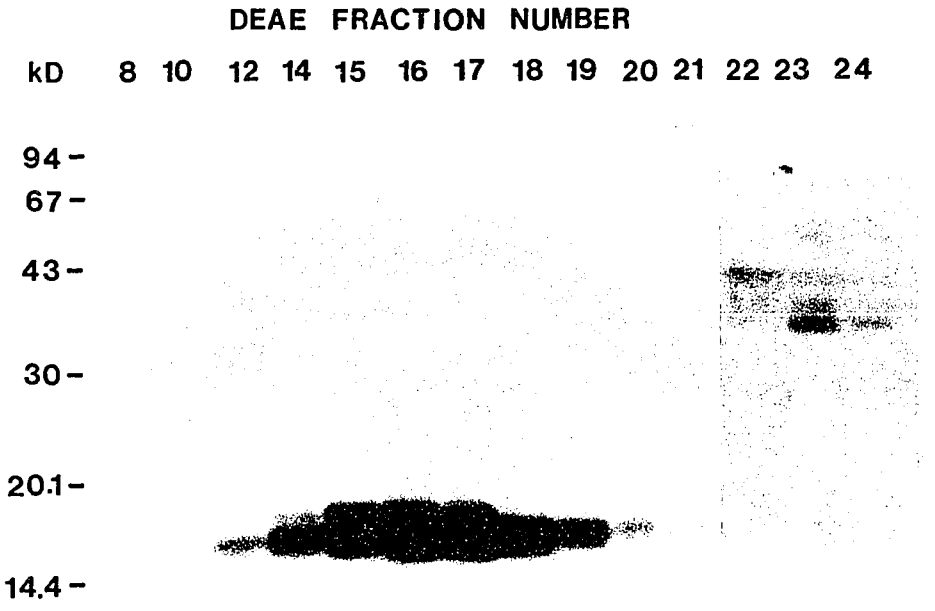

Figure 1. Autoradiograph of *In situ* phosphorylated fractions eluted from DEAE-sepharose column. Each lane was loaded with 10μL of eluate in 2X LSB and separated on 12.5% acrylamide gel with 100μg mL^{-1} histone H1 in the matrix.

separated into two phosphorylating proteins at 16.5kD and 18kD, the lower band being more heavily represented (Fig 1). No other autophosphorylating proteins were present in these fractions, however, several high molecular mass phosphorylating proteins were detected in subsequent fractions corresponding to conductivity 25 to 30 mS cm^{-1} (Fig 1).

Peak fractions with pp18 activity were pooled, diluted 5-fold to adjust the salt concentration and chromatographed directly on ATP-agarose column. Elution of the column with 1M NaCl (pH4) released small amounts of pp18 with some higher molecular mass protein impurities. The majority of pp18 was eluted as two bands at 16.5kD and 18kD into the 0.5mM GDP fraction with no other contaminating proteins as detected by silver staining (Fig 2A, lane 3). Both 16.5kD and 18kD proteins retained the ability to autophosphorylate *in situ* on histone gels (Fig 2B, lane 3).

The pp18 purified by the present protocol contained several isoforms at 16.5kD with isoelectric points (pI) 5.2, 5.5-5.6 and 5.9-6.0, and two isoforms at 18kD with pI 5.6 and 6.0, as detected by silver staining after two dimensional IEF/SDS-PAGE (Fig 3A). These pI values matched those for the previous preparations of pp18 (Moisyadi, 1991). All the isoforms

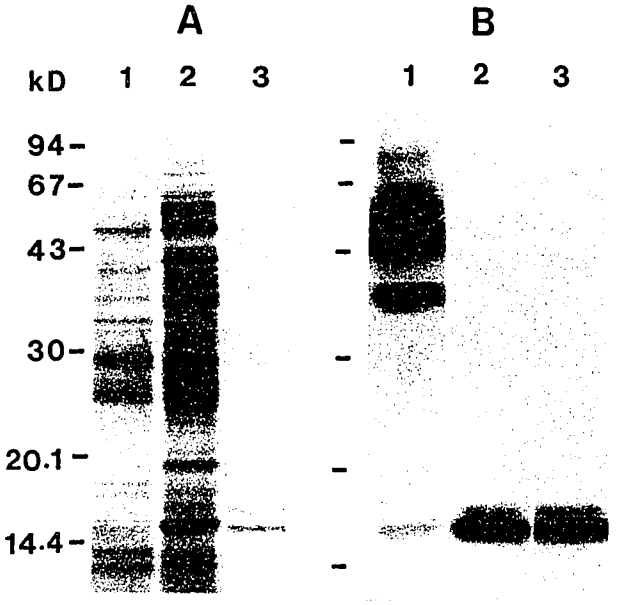

Figure 2. Purification of pp18. Panel A: silver stained gel; Panel B: autoradiograph of *in situ* phosphorylated proteins. Lanes 1, crude extract; 2, DEAE-sepharose peak fraction; 3, 0.5mM GDP eluate from ATP-agarose column.

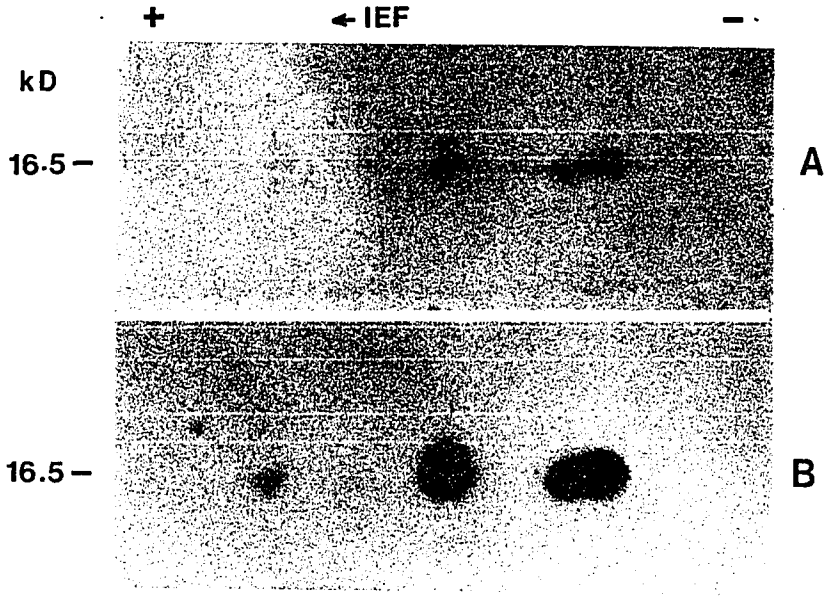

Figure 3. Two dimensional IEF/SDS-PAGE of affinity purified pp18 (0.5mM GDP eluted proteins from ATP-agarose column). Panel A: silver stained gel, Panel B: autoradiograph of *in situ* phosphorylated proteins.

phosphorylated *in situ* on histone gels (Fig 3B). The two major isoforms at pI 5.6 and 6.0 correspond to the low molecular mass phosphorylating proteins detected in crude extracts (Moisyadi, 1991). These results suggest the presence of multiple isoforms or isozymes of pp18, some of which were below the level of detection in crude extracts. Based on the autophosphorylation assay, the present protocol resulted in approximately 840-fold purification of pp18 (Table 1).

Table 1. Purification of pp18 from sugarcane cells.

Purification step	Total protein(mg)	Specific activity ^{32}P incorporation pmol mg^{-1} min^{-1}	Purification
Crude extract	1058	0.06	---
DEAE-sepharose peak fractions	74.5	1.9	32
ATP-agarose GDP eluate	0.28	50.4	840

Table 2. Summary of automated Edman degradation of the pp18 protein. Amino acids in parentheses were identified in the Edman cycle at a level of at least 50% of the major amino acid.

Edman cycle	Run 1 pmol	Run 2 pmol	Run 3 pmol
1	Val 3.0	Val 3.7	Val 2.7
2	Lys 1.8	Lys 0.6	Lys 0.9
3	Pro 3.2	Pro 1.2	Pro 1.7
4	Asp 2.8	Asp 0.9 (Gly)	Asp 1.9 (Gly)
5	Gly 1.3	Gly 1.8	Gly 0.8
6	Val 2.2	Ser 1.3	Val 1.7 (Ser)
7	Gln 1.7 (Val)	Gln 0.6 (Val)	Gln 1.2 (Val)
8	Arg 2.7	Arg 0.3	Arg 0.3
9	Gly 1.1 (Pro)	Gly 0.3	Gly 0.5
10	Leu 1.0	Leu 0.9	Leu 0.3
11	Xaa*	Xaa	Xaa
12	Gly 0.9	Gly 0.9	Xaa
13	Glu 0.8	Glu 0.3	Xaa
14	Ile 0.6	Val 0.3	Xaa

* Xaa, unknown amino acid

Protein sequence analysis. Table 2 summarizes the amino acid sequence analysis data of the pp18 protein CNBr fragment. Even though the same major sequence was present in each of three independent sequence runs, there was always a persistent background of amino acids in each cycle. The disparity of amino acids in cycles 4, 6, 7, 9 and 14 suggests the presence of isoforms.

A search of the Protein Information Resource database (Release 33) using FASTA (Pearson and Lipman, 1988) resulted in NDP kinase from various organisms, exhibiting up to 85% identity with a conserved region of other NDP kinases and homologs (Fig 4). The presence of multiple isoforms of NDP kinases in many organisms may explain the sequence disparity observed here. In mammals these isoforms are only slightly different in molecular masses (17.1kD and 17.29kD) (Gilles et al., 1991), while in spinach the isoforms show molecular masses of 16kD (NDP kinase I) and 18kD (NDP kinase II) (Nomura et al., 1991).

Characterization of NDP kinase activity. The purified pp18 exhibited NDP kinase activity as determined by the transfer of ^{32}P labelled γ-phosphate group from ATP to GDP, forming labelled GTP (Fig. 5A). This reaction was linear with time for about 15 min and with amount of enzyme up to 10ng μL^{-1} (Fig. 5B,C). The reaction was independent of incubation temperature from 23 to

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Sugarcane pp18                  M-V-K-P-D-G-V-Q-R-G-L-x-G-E-I
Human Nm23-H1          1........A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Human Nm23-H2          1........A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Mouse NDPK             1........A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Slime mold NDPK 1.............A-:-:-:-:-:-:-A-:-:-:-V-:-:-:
Drosophila AWD        1.........:-:-:-:-:-:-:-:-:-:-:-V-:-K-:
Spinach NDPK I            1.....:-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Spinach NDPK II       1.........:-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Rat NDPK α             1........A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
Rat NDPK β             1........A-I-:-:-:-:-:-:-:-:-:-V-:-:-:
E. coli NDPK           1........I-I-:-:-N-A-:-A-K-N-V-I-:-N-:
M. xanthus NDPK        1........I-I-:-:-:-:-L-E-K-:-V-I-:-K-:
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Figure 4. Sequence comparison of sugarcane pp18 CNBr digested fragment with known NDP kinases and Homologs. The number of amino acids preceeding the shown regions of each sequence are represented by a dot.

42, and also showed a broad pH range with an optimum at pH7.4. These characteristics of NDP kinase may aid its role in maintaining cellular energy levels under different environmental conditions.

Kinetic analysis of enzyme preparations that contained both molecular mass classes of NDP kinase showed apparent K_m values of 2.3mM and 0.2mM for ATP and GDP, respectively. Spinach NDP kinase I (M_r 16,000), which has K_m values 2.0mM and 0.091mM for ATP and GDP respectively (Nomura et al., 1991). Spinach NDP kinase II has a molecular mass of 18kD with K_m values of 0.89mM and 0.10mM for ATP and GDP, respectively (Nomura et al., 1991). In the preliminary preparations of sugarcane pp18, 16.5kD isoform was heavily represented over the 18kD isoform, thus may have contributed largely to the observed kinetic constants for the sugarcane NDP kinase preparation.

Terminology. In comparison with the spinach NDP kinases type I and II, the two molecular mass classes of sugarcane NDP kinases were termed NDP kinase I (16.5kD) and NDP kinase II (18kD).

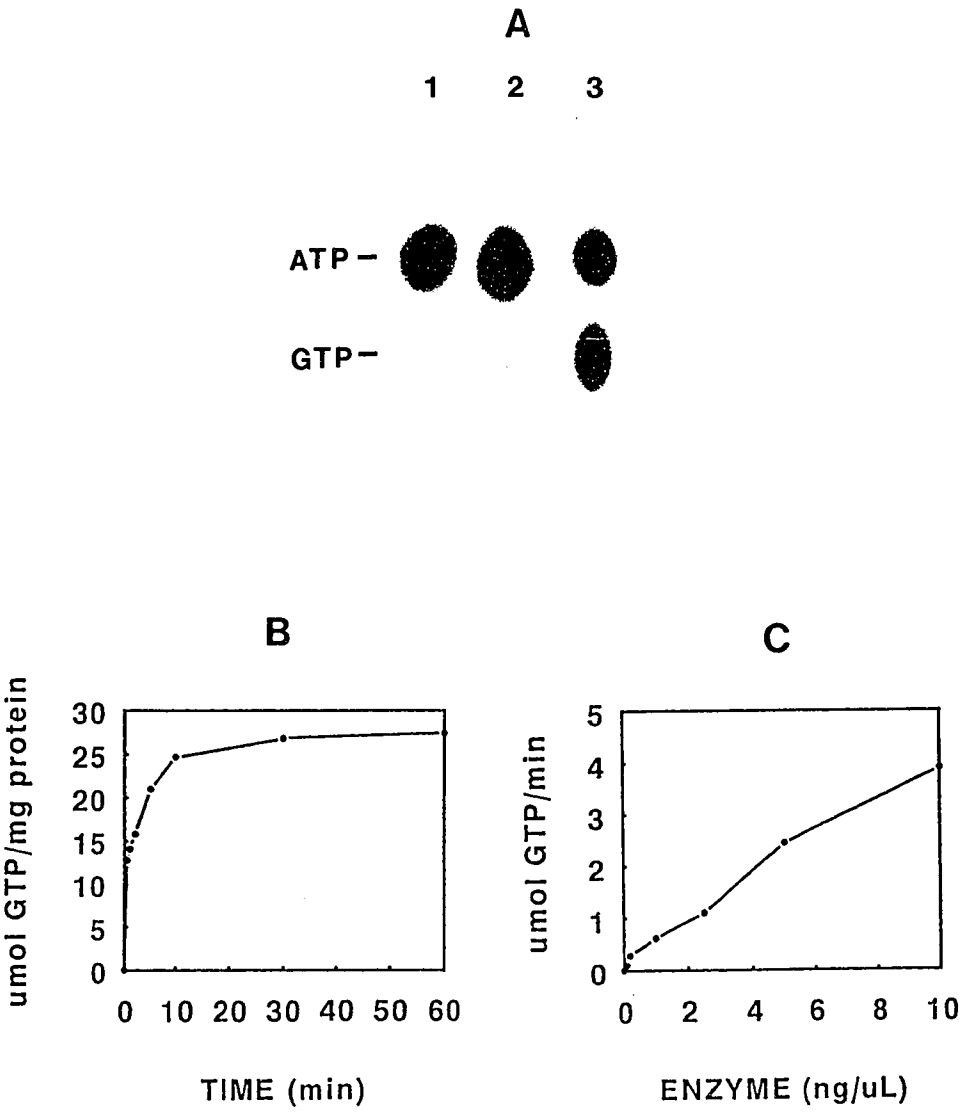

Figure 5. Analysis of NDP kinase activity in sugarcane pp18.
Panel A: Autoradiogram of TLC. Lane 1, no pp18; lane 2, with purified pp18; lane 3, with purified pp18 and BSA. Panel B: Time course of NDP kinase reaction. Panel C: Effect of protein concentration on NDP kinase reaction.

CHAPTER IV

MOLECULAR CLONING OF SUGARCANE NDP KINASE

INTRODUCTION

Genes or cDNAs encoding NDP kinases from various organisms ranging from bacteria to higher plants to humans have been cloned and sequenced. These include *Myxococcus xanthus* (Munoz-Dorado et al., 1990), *Escherichia coli* (Hama et al., 1991), *Dictyostelium discoideum* (Lacombe et al., 1990), rat (Ishikawa et al., 1992; Shimada et al., 1993), *Arabidopsis* (Quigley, 1992), spinach (Nomura et al., 1992; Zhang et al., 1993), rice (Yano et al., 1993), pea (Finan et al., 1994) and tomato (Harris et al., 1994). In *Drosophila*, the *awd* gene involved in normal development (Biggs et al., 1990) and in humans *nm23* genes controlling the tumor metastatic potential (Rosengard et al., 1989) are NDP kinase homologs. Based on their deduced amino acid sequences, NDP kinases appear to be highly conserved through evolution.

Nucleoside diphosphate kinases associate with different subcellular structures, form complexes with proteins and bind to certain DNA acting as a transcription factor suggesting a multifunctional nature of the protein. The NDP kinase activity associated with the cytosol, plasma membrane and microtubules may play a regulatory role in cellular energy metabolism involving non-adenine nucleotides, G-protein activation and cell motility

directly or indirectly by maintaining the available NTP levels (Parks and Agarwal, 1973; Walton and Gill, 1975; Kimura and Shimada, 1988; Wieland et al., 1991; Randazzo et al., 1992; Nickerson and Wells, 1983; Biggs et al., 1990; Melki et al., 1992). Additionally, the existance of mitochondrial (Herbert et al., 1955; Troll et al., 1993), nuclear (Biggs et al., 1990) and cell surface (Urano et al., 1993) localized NDP kinase homologs further suggest functions other than conversion of ATP to other NTPs.

Different isoforms of NDP kinase are encoded by separate nuclear genes. Mitochondrial and cytosolic NDP kinase homologs of *Dictyostelium discoideum* (Troll et al., 1993), α and β isoforms of rat NDP kinase (Shimada et al., 1993), H1 and H2 isoforms of human Nm23 proteins (Stahl et al., 1991) and type I and type II isoforms of spinach NDP kinases (Nomura et al., 1992; Zhang et al., 1993) are encoded by different genes which are evolutionarily conserved to a great extent within an organism as well as between organisms.

Purified sugarcane NDP kinase (pp18) separates into at least 5 isoforms of 16.5kD and two isoforms of 18kD on two-dimensional IEF/SDS-PAGE. The objective of the study presented here is to isolate cDNAs that encode different isoforms of sugarcane NDP kinases, and to deduce the amino acid sequences. The knowledge of the cDNA sequences and primary structures of

the isoforms may facilitate an understanding of the mode of regulation of NDP kinase isoforms during heat shock in sugarcane.

MATERIALS AND METHODS

Sugarcane cDNA library. A primary cDNA library of mRNA from cultured sugarcane cells (cell line H50-7209) constructed in λgt11 (Stratagene) was obtained from Dr. Paul A. Moore of Hawaii Sugar Planters Association (HSPA) Research Center. The primary library was amplified in *E. coli* Y1088 r^-m^- host cells and stored at 4°C. The titer of the primary library was 1.0 X 10^6 pfu mL^{-1}, and of the amplified library was 1.1 X 10^{11} pfu mL^{-1}.

Preparation of probe DNA. A 627bp cDNA (EcoRI digested) containing the entire coding region, and 5' and 3' untranslated regions of the spinach NDP kinase I (Nomura et al., 1992) was a gift from Dr. Atsushi Ichikawa of Kyoto University, Japan. The spinach cDNA was inserted into pBluescript KS- vector (Stratagene) at the EcoRI site and used to transform *E. coli* strain XL 1 Blue cells. Recombinants were selected based on blue/white color of colonies on agar plates with IPTG and X-gal. Plasmid DNA from a solitary recombinant bacterial colony was purified according to the $CsCl_2$ method of Sambrook et al. (1992). The purified plasmid DNA (20μg) was digested with Sau3AI and separated on 2% agarose gel. The band corresponding to a 414bp fragment

from 23 to 437bp of the coding region of spinach NDP kinase I (Nomura et al., 1992) was excised from the gel. The DNA was electroeluted using micro-eluter (Hoeffer Scientific, GE200) following manufacturers instructions and used as the probe for screening sugarcane cDNA library and subsequent experiments. The probe DNA was labelled with α-^{32}P-dCTP using random primers DNA labelling kit (BRL) according to the manufacturers instructions.

Southern hybridization of sugarcane genomic DNA. Genomic DNA from cultured sugarcane cells was isolated according to the method of Rogers and Bendich (1994), and 10μg total genomic DNA was digested with either EcoRI or HindIII. The digested DNA was separated on a 0.8% agarose gel and transferred onto nylon membrane (MSI) with 10X SSC for 16 h at room temperature (Southern, 1975). The blot was washed briefly with 10X SSC, air dried and UV cross linked. Probe DNA was prepared by labelling the 627bp spinach NDP kinase I cDNA (-33 to 594bp) using random primers DNA labelling kit (BRL). Prehybridization (2 h) and hybridization to probe DNA (18 h) were carried out at 60°C , the blot was washed and exposed to preflashed Fuji X-ray film (see appendix for compositions of hybridization and washing solutions).

Screening cDNA library. *Escherichia coli* strain Y1090 host cells were grown to an optical density (600nm) of 0.5 in LB medium containing 0.2% maltose and 10mM $MgSO_4$. Aliquots (55,000 pfu per plate) of amplified cDNA library were plated on 150mm petri dishes containing NZY bottom agar with *E.coli* strain Y1090 host cells and NZY top agarose. The plaque DNAs were transferred onto nylon membrane, denatured and fixed according to the method of Benton and Davis (1977). By screening $1.2X10^6$ plaques, 25 individual plaques were picked up as positive clones. After the secondary and tertiary screening, 21 clones were selected as hybridizing with spinach NDP kinase I cDNA (Sau3AI digested 414bp fragment) as a probe. Solitary plaques from 21 positive clones were bored out with bottom agar and placed in 0.5mL of SM medium with few drops of chloroform. The phage stocks were stored at 4°C.

Isolation and subcloning of phage DNA from positive clones. Phage DNAs were purified from all 21 clones according to miniprep liquid lysate method of Sambrook et al. (1992), restriction digested with EcoRI and separated on 2% agarose gel. The DNA was transferred onto nylon membrane and hybridized with spinach NDP kinase I cDNA probe as described above to visualize the sizes of cDNA inserts. Four independent inserts were subcloned into pBluescript KS- vector at EcoRI site and used to transform *E. coli* XL 1 Blue host cells as described before.

Sequencing cDNA inserts. Plasmid DNA from four cDNA clones in pBluescript vector were purified using BioRad Prep-A-Gene miniprep DNA isolation kit, according to manufacturers instructions. The cDNAs were sequenced at the Biotechnology Facility of the University of Hawaii, using automated sequencing apparatus.

RESULTS AND DISCUSSION

Southern hybridization. Results of the Southern hybridization of spinach NDP kinase I cDNA probe with sugarcane genomic DNA are shown in figure 6. EcoRI and HindIII digestions of genomic DNA resulted in single major bands at 660bp and 520bp respectively. Longer exposures revealed additional minor bands of larger sizes, 2.7 and 2.9 kb respectivly in EcoRI and HindII digestions. These results suggest that in sugarcane NDP kinase I may be encoded by a highly conserved small gene family. The small sizes of the only major bands in both restriction digestions further indicate that sugarcane NDP kinase gene (or genes) may not contain large introns.

cDNA library screening. A sugarcane cDNA library in λgt11 vector was screened using a 400bp cDNA fragment from the coding region of spinach NDP kinase I. After three rounds of screening for positive signals, 21 clones were selected as hybridizing strongly to the spinach NDP kinase I cDNA.

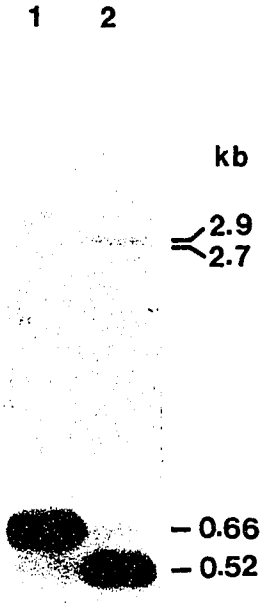

Figure 6. Southern hybridization of sugarcane genomic DNA with spinach NDP kinase I cDNA. Lane 1, EcoRI digestion; lane 2, HindIII digestion

Insert sizes of these 21 individual phages are shown in Table 3. The sizes indicated that several clones were either identical or had sequences of the same length. The few large sequences (i.e pSCNDK1, 15 and 21) could be either cloning artifacts or unrelated sequences with some homology to spinach NDP kinase I sequence. Four inserts in phage vector were subcloned into pBluescript vector for further analysis; pSCNDK5 (0.75bp), pSCNDK8 (0.78bp), pSCNDK10 (0.75bp) and pSCNDK19 (0.62bp). The pBluescript plasmids harboring the cDNAs were purified and aliquots were digested with EcoRI. The digested DNAs were separated on 2% agarose gel to visualize the inserts (Figure 7). The cDNAs were sequenced using standard T3, T7 and M13 forward primers and sequence specific internal primers.

cDNA clone pSCNDK8; sequence and deduced amino acid sequence. The cDNA clone pSCNDK8 contained the entire coding region of sugarcane NDP kinase I of 447bp with a 58bp 5' flanking sequence and 238bp 3' flanking sequence (Figure 8). The actual length of the cDNA (743bp) was less than the predicted length of the clone as detected on the agarose gels (780 bp). This anomaly may be due to the differential migration of DNA and molecular weight markers on the gel. The first ATG codon at the positions 1 to 3 of the cDNA sequence was considered as the initiation codon. This open reading frame terminated at a TAA codon beginning at the position 448. Thus, the cDNA

Table 3. Insert sizes of the cDNA clones hybridizing to spinach NDP kinase I cDNA probe.

Clone (pSCNDK--)	Insert size (kb)
1	3.99
2	1.76
3	1.64
5	0.75
6	0.73
7	0.75
8	0.78
9	0.62
10	0.73
11	0.75
13	1.84
15	4.82
16	0.75
17	0.75
18	0.75
19	0.62
21	5.57
22	0.75
23	0.78
24	0.75
25	0.73

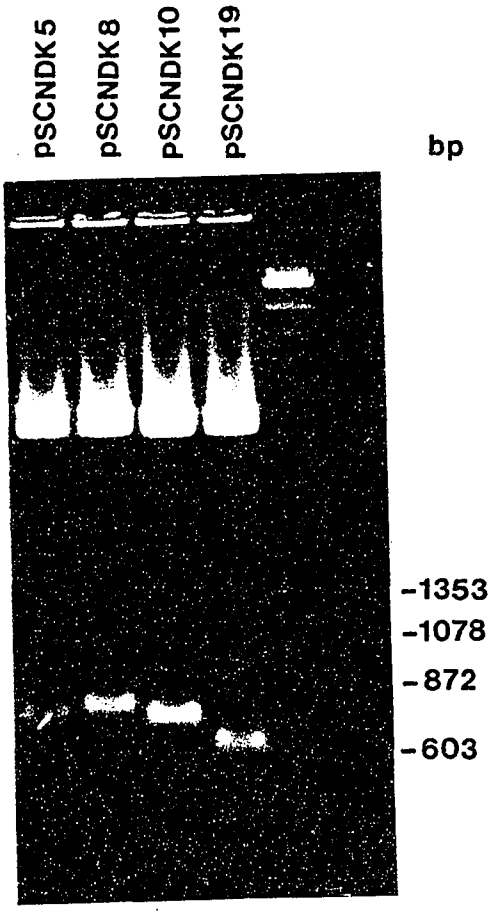

Figure 7. Agarose gel electrophoresis of EcoRI digested pBluescript plasmid DNA harboring four cDNA inserts that react with spinach NDP kinase cDNA.

```
1       .       .       .       .       .       .
tcaaaaaccagaccaaaccccccgaactaggagccagcgcccgcttgcagtagccgcaat
                                                      M

61      .       .       .       .       .       .
ggagagcacctttatcatgatcaagcctgacggcgtccagaggggcctcattggtgagat
 E  S  T  F  I  M  I  K  P  D  G  V  Q  R  G  L  I  G  E  I

121     .       .       .       .       .       .
cattagccgtttcgagaagaagggcttctacctcaaggccttgaagcttgtgaacgtgga
 I  S  R  F  E  K  K  G  F  Y  L  K  A  L  K  L  V  N  V  E

181     .       .       .       .       .       .
gaggtcgttcgccgagaagcactacgctgatctatcctccaagcccttcttccagggcct
 R  S  F  A  E  K  H  Y  A  D  L  S  S  K  P  F  F  Q  G  L

241     .       .       .       .       .       .
cgtggactacatcatctctggccctgtggtggccatggtctgggagggcaagagcgtcgt
 V  D  Y  I  I  S  G  P  V  V  A  M  V  W  E  G  K  S  V  V

301     .       .       .       .       .       .
cacaactggccgcaagatcatcggggccaccaacccctggtttctgagcccggcaccat
 T  T  G  R  K  I  I  G  A  T  N  P  L  V  S  E  P  G  T  I

361     .       .       .       .       .       .
ccgtggcgactttgtgttcgacattgccaggaatgtcattcatggaagtgacagcattga
 R  G  D  F  V  F  D  I  A  R  N  V  I  H  G  S  D  S  I  E

421     .       .       .       .       .       .
gagtgctaacaaggagattgctctgtggttccccgagggcctcgctgattggcagagcag
 S  A  N  K  E  I  A  L  W  F  P  E  G  L  A  D  W  Q  S  S

481     .       .       .       .       .       .
ccagcacccctggatctacgagaagtaaaacgttgaatgtttcacattcaaccttgagag
 Q  H  P  W  I  Y  E  K

541     .       .       .       .       .       .
ccagaggaagcatggcaccacctgtgctttagcaatatttagagtttgaaaagattatga

601     .       .       .       .       .       .
tctgtatctcaaccttatttccgtgggccgaatttccttccttgccttgcatgttattca

660     .       .       .       .       .       .
tgcttacattttgcttttgaattttatatggatatggtattgatttcatgaggtacccca

721     .       .
agcaccccaactcaaaaaaaaaa
```

Figure 8. cDNA sequence and deduced amino acid sequence of pSCNDK8.

codes for a 149 amino acid sequence with a predicted molecular mass of 16,415 daltons. This size was in agreement with the purified sugarcane NDP kinase I which resolved at 16.5kD upon SDS-PAGE (see figures 2A & 3A, Chapter III).

The deduced amino acid sequence of pSCNDK8 showed a high sequence identity and similarity with many known eukaryotic NDP kinases (Figure 9), the highest identity being with *Pisum sativum* NDP kinase (86%). The similarities between pea and sugarcane sequences were 88%. The sugarcane sequence also showed 84% and 74% identities, and 88% and 78% similarities with spinach NDP kinase I and *Arabidopsis thaliana* NDP kinase, respectively. Interestingly, the sequence identity and similarity between sugarcane pSCNDK8 and rice NDP kinase, another available sequence from a monocot species, were 74.4% and 82%, respectively. The striking evolutionary conservation of NDP kinase sequences between animals, dicots and monocots further exemplifies the essential role of this enzyme in these organisms.

The regions involved in NDP kinase catalytic activity and other conserved domains were analysed on the primary amino acid sequence. The glycine rich consensus sequence 'G-X-G-X-X-G' is a motif common to nucleotide binding proteins and protein kinases (Hanks et al., 1988) and

```
SCNDK8    M-------ESTFIMIKPDGVQRGLIGEIISRFEKKGFYLKALKLVNVERSFAEKHYA
PSNDKP1   *--------*Q**************V***************G**F*****A*******
SPNDPKI   *--------*Q**************V************S*****F***D*P*******
ATNDK     *--------*Q***************VSVKS*AGLRRRVS*-C***IS**********E
OSNDPK    *--------*Q****************D************RGM*FM*******QQ****
DMAWD     *AANK---*R*****V*********V*K**E***Q***K*V***FTWASKELL*****
NM23-H1   *A-NC---*R***A**********V****K***Q***R*VG**FMQASEDLLKE**V
DDNDPK    *STNKVNK*R*LAV******A***V****A*Y*****V*VG**QLVPTKDL**S***
MXNDPK    AI-------*R*LSI*****LEK*V*GK******E**LKPV*IR*QHLSQ*Q**GF**

SCNDK8    DLSSKPFFQGLVDYIISGPVVAMVWEGKSVVTTGRKIIGATNPLVSEPGTIRGDFVF
PSNDKP1   ***A****S***************I****N***************AQ**********AI
SPNDPKI   ***A****N***E**V************G**A****L*******A**********AI
ATNDK     *****S**S******V********I****N**L***********AA**********AI
OSNDPK    ***D****P***E***************D**A***R*****R*WEAA*****A*YAV
DMAWD     ***AR***P***N*MN*****P*****LN**K***QML*****AD*L********CI
NM23-H1   **KDR***A***K*MH***********LN**K***VML*E***AD*K********CI
DDNDPK    EHKER***G***SF*T********F***G**ASA*LM**V****A*A**S*****GV
MXNDPK    VHKAR***KD**QFM******L**L**ENA*LAN*D*M*****AQAAE****K**AT

                                                          %iden %simil
SCNDK8    DIARNVIHGSDSIESANKEIALWF-PEGLADWQSSQHPWIYEK      ---   ---
PSNDKP1   **G********AV**********-***A*N*E**L*S****-      85.9  87.9
SPNDPKI   **G********AVD**T*******-*D*VVH****L*S****-     81.8  88.5
ATNDK     **G*********V***R*******-*D*PVN****V***V**T      74.0  81.2
OSNDPK    EVG*********VDNGK*******-*****E*R*NL******S      74.4  82.0
DMAWD     QVG**I*****AV***E*******NEKE*VT*TPAAKD****-      61.4  67.3
NM23-H1   QVG**I******V***E*******H**E*V*YT*CAQN****-      61.8  65.7
DDNDPK    *VG**I******V****R******K**E*LT-EVKPN*NL**-      58.3  64.7
MXNDPK    S*DK*TV*****L*N*KIE**YF*RETEIHSYP--------*Q*     47.0  51.0
```

Figure 9. Comparison of sugarcane SCNDK8 deduced amino acid sequence with known NDP kinases. PSNDKP1 = *Pisum sativum* NDP kinase; SPNDPK1 = spinach NDP kinase I; ATNDK = *Arabidopsis thaliana* NDP kinase; OSNDPK = *Oryza sativa* NDP kinase; DMAWD = *Drosophila melanogaster* AWD protein; NM23-H1 = human Nm23 protein; DDNDPK = *Dictyostelium discoideum* NDP kinase; MXNDPK = *Myxococcus xanthus* NDP kinase. Percentage identities and similarities are given at the end of each sequence.

suggested as the putative nucleotide binding domain in spinach NDP kinase (Nomura et al., 1992). In the SCNDK8, sequence G^{12}-V-Q-R-G^{16}-L-I-G^{19}, which is identical in rice and *Myxococcus xanthus* NDP kinase may be the putative nucleotide binding domain. In all the other known NDP kinase sequences this region is identical except the presence of a valine instead of isoleucine[18]. Thus, conservation of this domain appears to be extremely high. However, the recent x-ray crystallographic analyses of the *Dictyostelium discoideum* and *Myxococcus xanthus* NDP kinases shows that the glycines in this region did not show a direct interaction with the nucleotide (Morera et al., 1994; Williums et al., 1993). Based on the crystallographic data, Williums et al. (1993) suggest that the conserved glycines may stabilize the structure of the protein facilitating nucleotide binding rather than having a direct effect. In addition, the conserved G^{89} in a motif present in various GTP binding proteins (Munoz-Dorado et al., 1993) is suggested to be involved in nucleotide binding. In *Myxococcus xanthus*, substituting G^{91} with a valine completely abolishes the NDP kinase activity as well as autophosphorylation (Munoz-Dorado et al., 1993). However, subsequent x-ray structure shows no direct interaction between this glycine and the bound nucleotide suggesting an indirect effect (Williums et al., 1993).

The proposed NDP kinase catalytic site amino acid sequence was identical in sugarcane sequence and in all the known NDP kinase sequences. The conserved histidine residue at position 118 in human erythrocyte NDP kinase is shown to be the phosphorylated residue in phosphoenzyme intermediate (Gilles et al., 1991). In the sugarcane sequence, the amino acid residues corresponding to H^{114}-G-S-D may be the catalytic site for NDP kinase activity, H^{114} being the phosphorylated residue. The conserved lysine residue at position 36 of the sugarcane sequence may also be important in catalytic activity of the NDP kinase reaction. This lysine residue may be involved in the phosphotransfer reaction, probably by mediating the proton transfer (Kamps and Sefton, 1986).

In addition to the phosphorylated enzyme intermediate, NDP kinases undergo serine phosphorylation (MacDonald et al., 1993; Hemmerich and Pecht, 1992) via inter- or intra-molecular phosphate transfer from the phosphorylated histidine (Munoz-Dorado et al., 1993; MacDonald et al., 1993). In human Nm23-H1, a serine residue at position 44 is autophosphorylated as shown by acid hydrolysis and proteolytic digestion of the phosphorylated enzyme (MacDonald et al., 1993). This serine phosphorylation is also coupled with the metastatic suppressor activity of the human Nm23 (MacDonald et al., 1993). In sugarcane pSCNDK8 sequence, a serine residue is present at position

43, and may be a site of acid-stable autophosphorylation as demonstrated for the purified sugarcane NDP kinase (Moisyadi et al., 1994).

Interestingly, the deduced amino acid sequence for pSCNDK8 differs from the peptide sequence generated in protein sequence analysis after CNBr digestion (figure 9). This discrepancy may be due to the presence of multiple isoforms of NDP kinase in sugarcane. However, subsequent sequencing of three other cDNAs did not show a precise sequence identity with the CNBr digested peptide sequence (see below).

cDNA clones pSCNDK5, pSCNDK10 and pSCNDK19; nucleotide and deduced amino acid sequences. As shown in figure 10 A, three other cDNA clones sequenced in this study had a very high identity to pSCNDK8 (approximately 97%) at the nucleotide level, especially in the coding region. The variations indicated that these different clones were encoded by different genes belonging to a highly conserved family. The base differences in the coding region were mostly at the 3rd position of codons thus did not affect the amino acid residue except in a few instances. The base pair differences were more frequent in the non-coding regions, especially in the 3' flanking sequence.

The clone pSCNDK19 showed the highest variation at nucleotide level as compared to pSCNDK8. pSCNDK19 deduced amino acid sequence was one residue shorter than the other sequences due to a substitution of 'aag' of the last codon of the reading frame with a terminating 'tag' codon. Thus in this sequence the terminal lysine residue was absent (figure 10 B). The 3' flanking region of pSCNDK19 was considerably shorter than that of other sequences (95bp, in contrast to 238bp in pSCNDK8), however, if this variation was due to actual differences in the mRNA or due to cloning artifacts is currently not clear.

The clone pSCNDK5 and pSCNDK10 showed a remarkable sequence identity to pSCNDK8 at the nucleotide level (figure 10 A). However, in pSCNDK5, a two base pair addition causes a frame shift in the deduced amino acid sequence completely deminishing the identity with NDP kinases after the 97th residue of the amino acid sequence. This region includes the conserved NDP kinase domain with the histidine residue which catalyzes the transfer of phosphate group during NDP kinase enzyme activity (figure 10 B). Thus, the modified protein may not be functioning as an NDP kinase in the cells. However, due to the presence of other conserved regions at the amino terminus, it may be performing some other functions attributed to NDP kinases (see chapter I). The deduced amino acid sequence of pSCNDK5 had 152

residues (predicted molecular mass of 16,745 daltons), closely matching the size characteristic to NDP kinases studied so far in various organisms (see chapter I). If this protein is synthesized *in vivo* in sugarcane cells is currently not known.

Based on the sequence data of the cDNA clones it can be suggested that sugarcane NDP kinase I proteins are encoded by different genes, and may be functionally distinct. It is possible that these different isoforms are sequestered in separate cellular compartments as suggested previously for NDP kinases in other organisms (Troll et al., 1993; Shimada et al., 1993). The present study did not detect any cDNAs encoding sugarcane NDP kinase II isoforms. Three cDNAs in the range of 1.64-1.84kb (pSCNDK2,3 and 13) matched the possible size of NDP kinase II cDNA sequence based on the size of spinach NDP kinase II (Zhang et al., 1993). Sequencing pSCNDK2 cDNA revealed a 600bp carboxyl terminal portion of NDP kinase I cDNA including poly(A) tail attached to another cDNA at the 3' end that dit not show homology to any known NDP kinase sequences (data not shown). However, this does noe exclude the possibility that one or both of the other 2 cDNAs (pSCNDK3 and 13) may encode sugarcane NDP kinase II proteins.

```
          1                                                    50

scndk8    ..tcaaaaac cagaccaaac cccccgaact aggagccagc gcccgcttgc
scndk5    ...t***c** ********** ********** ********** **********
scndk10   .......... ..t******* *********c ********** **********
scndk19   tga******** ********** *********c ********** **g*******

          51                                                   100

scndk8    agtagccgca atggagagca cctttatcat gatcaagcct gacggcgtcc
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ****c***** ********** **********
scndk19   ********** ********** ****c***** ********** **********

          101                                                  150

scndk8    agaggggcct cattggtgag atcattagcc gtttcgagaa gaagggcttc
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ********** ********** ********** ********** **********

          151                                                  200

scndk8    tacctcaagg ccttgaagct tgtgaacgtg gagaggtcgt tcgccgagaa
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ********** ********** ********** ********** **********

          201                                                  250

scndk8    gcactacgct gatctatcct ccaagccctt cttccagggc ctcgtggact
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ********** ********g* ********** ********** **********


          251                                                  300

scndk8    acatcatctc tggccctgtg gtggccatgg tctgggaggg caagagcgtc
scndk5    ********** ********** ********** ********** **********
scndk10   ********t** ********** ********** ********** **********
scndk19   ********** ********** ********** ********** **********

          301                                                  350

scndk8    gtcacaactg gccgcaagat catcggcgcc accaaccccc tggtttct..
scndk5    ********** ********** ********** ********** ********ga
scndk10   ********** ********** ********** ********** ********..
scndk19   **t******** ********** ********** ********** ***c**ct..
```

Figure 10. Comparison of cDNA clones pSCNDK5, pSCNDK8, pSCNDK10 and pSCNDK19. A. Nucleotide sequences; B. Deduced amino acid sequences.

```
scndk8    gagcccggaa ccatccgtgg cgactttgct gtcgacattg gaaggaatgt
scndk5    ********** ********** ********** ********** *c********
scndk10   *****t**** ********** ********** ********** **********
scndk19   ********** ********** t********* ********** *c********

          401                                                 450

scndk8    catccatgga agtgacagca ttgagagtgc taacaaggag attgctctgt
scndk5    ***t****** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ***t****** **c******* ********** ********** *****c****

          451                                                 500

scndk8    ggttccccga gggcctcgct gattggcaga gcagccagca cccctggatc
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ********** ********** ********** ********** **********

          501                                                 550

scndk8    tacgagaagt aaacgttgaa tgtttcacat tcaaccttga gagccagagg
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** *.********
scndk19   **t***t*** *****.g*g* *****t**** *g******** **a******t*

          551                                                 600

scndk8    aagcatggca ccagctgtgc tttagcaata tttagagttt gaaaagatta
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   ******a*** ********** ********** ********** **********

          601                                                 650

scndk8    tgatctgtat ctc..aacct tatttccg.t gggccgaatt tccttccttg
scndk5    ********** ***..***** ********.* **a******* **********
scndk10   ********** ***ag***** ********t. **a******* **********
scndk19   ***..... .......... .......... .......... ..........

          651                                                 700

scndk8    ccttgcatgt tattcatgct tacattttgc ttttgaattt tatatggata
scndk5    ********** ********** ********** ********** **********
scndk10   ********** ********** ********** ********** **********
scndk19   .......... .......... .......... .......... ..........

          701                                                 749

scndk8    tggtattgat ttcatgaggt accccaagca ccccaactca aaaaaaaaa
scndk5    ********** **aaaaaaaa aa........ .......... .........
scndk10   *****g**** **caaa.... .......... .......... .........
scndk19   .......... .......... .......... .......... .........
```

Figure 10 A. continued.

```
          1
 scndk8   MESTFIMIKP DGVQRGLIGE IISRFEKKGF YLKALKLVNV
 scndk5   ********** ********** ********** **********
scndk10   ********** ********** ********** **********
scndk19   ********** ********** ********** **********

          41
 scndk8   ERSFAEKHYA DLSSKPFFQG LVDYIISGPV VAMVWEGKSV
 scndk5   ********** ********** ********** **********
scndk10   ********** ********** ********** **********
scndk19   ********** ********** ********** **********

          81
 scndk8   VTTGRKIIGA TNPLVSE..P GTIRGDFAVD IGRNVIHGSD
 scndk5   ********** ****A**SPA PSVATLLSTL A*MSFMEVTA
scndk10   ********** *******..* ******LV** **********
scndk19   ********** ****A**..* ********** **********

          121
 scndk8   SIESANKEIA LWFPEGLADW QSSQHPWIYE K.
 scndk5   LRVLTRRLLC GSPRAS*IGR AA*TPGSTRS *R
scndk10   ********** ********** ********** *.
scndk19   ********** ********** ********** ..
```

Figure 10 B. Deduced amino acid sequences of pSCNDK clones.

CHAPTER V

SYNTHESIS, PHOSPHORYLATION AND ENZYME ACTIVITY OF NDP KINASE IN RESPONSE TO HEAT SHOCK

INTRODUCTION

Sublethal heat shock temperatures modulate the pattern of protein synthesis, protein modification as well as their functions. As discussed in chapter I, the HSR includes the induction or enhancement of a subset of proteins known as HSPs accompanied by the dramatic reduction in the synthesis of constitutive proteins. Cultured tobacco cells present a typical example for this general pattern of HSR (Kanabus et al., 1984; Harrington and Alm, 1988; Dharmasiri, 1990). In contrast, cultured sugarcane cells and maize tissues are among the few exceptions in that normal protein synthesis is unaffected by HS. In these organisms, the pattern of HSP synthesis is superimposed on the normal protein synthesis pattern (Moisyadi and Harrington, 1989; Moisyadi, 1991; Cooper and Ho, 1983; Mansfield and Key, 1987).

Proteins undergo several forms of post-translational modifications in response to HS. Phosphorylation and dephosphorylation are among the most common modes of regulation of protein functions during HS (Schlesinger, 1988). Phosphorylation and dephosphorylation of these proteins are controlled

by HS induced or activated protein kinases and phosphatases often acting as components of regulatory cascades leading to cellular response to the HS signal. For example, HS induces the autophosphorylation of HSP90 which in turn stimulates the induction of eIF2α kinase in HeLa cells (Duncan and Hershey, 1984). Phosphorylation of initiation factor eIf2α by this kinase negatively regulates the initiation of protein synthesis during HS (Duncan and Hershey, 1984). Phosphorylation of HSF upon HS plays a regulatory role in HS gene transcription (Sorger et al., 1987; Sorger and Pelham, 1988, Nieto-Sotelo et al., 1990).

In cultured sugarcane cells, a low molecular mass protein undergoes alterations in the ability to autophosphotylate *in situ* when subjected to 36°C HS for 2 h (Moisyadi, 1991). This protein has many properties identical to a low molecular mass phosphoprotein characterized as a protein kinase catalytic subunit designated pp18 present in pea bud plasma membrane (Blowers and Trewavas, 1988; 1989). The pp18 in sugarcane phosphorylates on serine residues and shows a rapid turnover of phosphate group (Moisyadi, 1991). Additionally, *in vivo* ^{35}S labelling of sugarcane cells indicates that two minor protein spots induced during HS, and corresponded to the major phosphorylation signals based on molecular mass and isoelectric points on two dimensional gels (Moisyadi, 1991). These observations suggest that the

synthesis of pp18 in sugarcane cells is induced or enhanced during HS. However, more specific experiments are needed to clarify this idea.

As discussed in chapters III and IV, the sugarcane pp18 was identified as an NDP kinase. This enzyme has been purified and characterized from a variety of organisms including plants, animals and bacteria (Kimura and Shimada, 1988; Prescan et al., 1989; Buczynski and Potter, 1990; Munoz-Dorado et al., 1990; Jong and Ma, 1991; Nomura et al., 1991 and White et al., 1993). Additionally, the genes and cDNAs encoding NDP kinases have been cloned and characterized from several eukaryotic and prokaryotic sources as discussed in chapter IV, however, only one report describes the induction of the NDP kinase transcript levels in response to a stress signal, in this case wounding (Harris et al., 1994).

Nucleoside diphosphate kinases mediate the enzymic reaction via a phosphorylated enzyme intermediate (Parks and Agarwal, 1973). The phosphorylated residue is usually a histidine (Gilles et al., 1991; Dumas et al., 1992; Munoz-Dorado et al., 1993), however, aspartyl or glutamyl residue has also been suggested as the phosphoenzyme intermediate (Hemmerich and Pecht, 1992). Additionally, NDP kinases undergo autophosphorylation at serine residues at a low stoichiometry, via an inter- or intra-molecular phosphate

transfer reaction (Hemmerich and Pecht, 1992; MacDonald et al, 1993; Munoz-Dorado et al., 1993). In Nm23-H1, an NDP kinase homolog in humans, this serine phosphorylation is important in controlling tumor metastatic potential, and also shown to be independent of NDP kinase enzyme activity (MacDonald et al., 1993). So far, there is no evidence for the regulation of the phosphorylation state or enzyme activity of NDP kinase in response to environmental stresses. The observation that sugarcane NDP kinase (pp18) undergoes changes in autophosphorylation during HS (Moisyadi, 1991) presents an important clue to the additional regulatory mechanisms for the sugarcane enzyme.

In this chapter, results are presented from the experiments conducted to investigate the mRNA and protein levels, phosphorylation and enzyme activity of NDP kinase in sugarcane cells in response to heat shock. A comparison of NDP kinase mRNA levels in intact sugarcane shoots, as well as phosphorylation and enzyme activity of NDP kinase in cultured tobacco cells in response to HS are included.

MATERIALS AND METHODS

Plant materials. Fourteen (14) day old suspension cultured sugarcane cells (*Saccharum officinaruml* L. cv. H50-7209), 4d old sugarcane shoots from the

same cultivar (cv. H50-7209) and 7d old suspension cultured tobacco cells (*Nicotiana tabaccum* L. cv. Wisconsin-38) were used in these experiments.

Polyclonal anti-NDPK I antibody. Rabbit polyclonal anti-serum raised against purified spinach NDP kinase I protein (Nomura et al., 1991) was a gift from Dr. Atsushi Ichikawa of Kyoto University, Japan.

Heat shock treatments and protein extraction from cultured sugarcane and tobacco cells for SDS PAGE. Suspension cultured sugarcane (14d old) and tobacco (7d old) cells were treated at 25, 34, 36, 38, 40 and 42°C for 2 hours in an orbital shaking waterbath. The cells (0.25g) were harvested, frozen and ground in liq. N_2, and extracted into 1mL of 2X Laemmli sample buffer (Laemmli, 1972) as described (Moisyadi and Harrington, 1989). Concentration of the proteins in the samples were assayed (Lowry et al., 1951).

Heat shock treatment and protein extraction from sugarcane cells at different culture ages. Sugarcane cells (10mL) from 14d old culture were transferred into 250mL Erlenmeyer flasks containing 70mL of growth medium and incubated in dark at 25°C on an orbital shaker. At each different culture age, two flasks were incubated at 25°C (control) or 38°C (HS) for 2 h, the cells were harvested as described before and frozen in liquid N_2. Total cellular

proteins were extracted into 2X Laemmli sample buffer as described previously and protein concentrations in the extracts were estimated (Lowry et al., 1951).

In situ phosphorylation of sugarcane and tobacco proteins. Total protein (20μg) were separated on 12.5% acrylamide gel containing 20μg mL histone H1 type IIIS in the matrix. After electrophoresis, the proteins were renatured and phosphorylated using γ-^{32}P-ATP as described before (phosphorylation method 1, Moisyadi et al., 1994). The gels were exposed to preflashed Fuji X-ray film, and bands corresponding to 16.5-18kD region were excised and the incorporation of radioactivity was measured using Beckmann liquid scintillation counter with 3mL of ScintiVerse E.

Heat treatments and isolation of total and poly (A) RNA from cultured sugarcane cells. Sugarcane cells (80mL cell suspension in 250mL Erlenmeyer flasks) were treated at 25, 34, 36, 38, 40 and 42°C for 2 hours in an orbital shaking waterbath, harvested as described before, and frozen in liquid N_2. Approximately 8 g of cells from each treatment were ground in liquid N_2 and total RNA were extracted according to the method of McGookin (1984). Poly (A) RNAs were isolated from 1 mg of total RNA from each sample using Poly A Tract (Promega) mRNA isolation kit, according to the manufacturers

instructions. All the solutions used for RNA extraction and subsequent manipulations were prepared with diethylpyrocarbonate (DEPC) treated water.

Heat treatments and isolation of total RNA from sugarcane shoots. Mature setts of sugarcane containing 2 internodes were soaked in 50° C water for 30 min, treated with fungicide (tilt, 40 μL L^{-1}) and planted on sterile vermiculite in 4X10 inch metal trays. The setts were watered moderately and placed in dark at 25°C with slight aeration. When the shoots were 1-1.5 inches long (4 day old), whole trays with plantlets were placed directly at 40°C for 30 min, 1 h, 2 h and 5 h in dark. The controls were directly harvested. A similar sample of shoots were wounded by puncture (6-8 punctures per shoot) with a sharp needle and placed at 25°C in dark for 5 hrs. At least 3 setts each containing 2 healthy shoots were used for each treatment. Approximately 1.5 g fresh weight of shoot tissue for each treatment were collected and ground in liquid N_2 to become a fine powder. Eight (8) mL of guanidine thiocyanate buffer was added into each sample and total RNA was extracted as before.

Northern hybridization. Denaturing 1.5% agarose gels containing formaldehyde were loaded with either 10μg per lane total RNA or 1μg per lane poly (A) RNA, with 9.5kb-0.24kb RNA ladder (BRL) as standard molecular weight marker. After electrophoresis with 1X MOPS buffer, the gel was

washed twice with DEPC treated water, 5 min each time, and finally with 10X SSPE for 15 min. The RNAs were transferred for 16 hours onto nylon membranes (MSI) using 10X SSPE. The blots were air dried for 5 min and UV cross-linked.

Northern hybridization was done using sugarcane cDNA clone pSCNDK8 as the probe (743bp insert was released from pBluescript by digestion with EcoRI and gel purified). The probe DNA was labelled with α-^{32}P-dATP using random priming DNA labelling kit (BRL) according to manufacturers instructions. The blots were prehybridized for 4 hours at 42°C and hybridized to probe DNA (approximately $3.5X10^7$ cpm total) for 30 hours at 42°C, the blots were washed and exposed to preflashed Fuji X-ray film for 12 h. (see appendix for hybridization and wash solutions)

Western blot analysis. Twenty (20) μg of total protein were separated on 12.5% acrylamide gels and transferred onto PVDF membrane (BioRad) at 50V (180mA) for 2 h at room temperature using a BioRad Mini Trans Blot transfer apparatus. The transfer buffer contained 0.1M CAPS (pH 11) and 10% methanol. The blots were blocked, washed (see appendix for blocking and washing reagents) and exposed to primary antibody (spinach NDP kinase I, polyclonal antisera). The bound antibodies were detected using alkaline

phosphatase conjugated goat anti-rabbit IgG second antibody (Sigma) and color development in the presence of BCIP and NitroBT.

Phenol extraction of proteins, two dimensional IEF/SDS-PAGE and western blot analysis. Fourteen (14) day old sugarcane cells were incubated at 25°C (control) or 40°C (HS) for 2 h and harvested as previously described. Half a gram (fresh weight) of cells from each treatment were frozen and ground in liquid N_2. The proteins were extracted according to the phenol partition method described by Hurkman and Tanaka (1987), and 50 μg equivalent of total proteins were separated by two dimensional IEF/SDS-PAGE as described previously (Moisyadi and Harrington, 1989). Proteins in the gels were transferred onto PVDF membrane and reacted with spinach NDP kinase I antibody as described above.

Immunoprecipitation of ^{35}S-labelled sugarcane proteins. Fourteen day old sugarcane cells (0.25g fresh weight in 1 mL culture medium) were transferred into sterile 50 mL tubes and placed at 25°C (control) or 40°C (HS). After 5 min, 800μCi of Tran-^{35}S-label (ICN Biomedicals) was added into each tube and incubated for further 2 h. At the end of the treatment the cells were filtered, washed thoroughly with 100 mL milli-Q H_2O and placed in 1.5 mL microfuge tubes. The tubes were rapidly dipped in a liq. N_2 bath and the cells were

ground using a douncer. Total sugarcane proteins were extracted into 0.5 mL of ice cold lysis buffer (50mM Tris pH8, 150mM NaCl, 1% NP-40, 1mM EDTA, 1mM PMSF, 5μg mL^{-1} Aprotinin and 0.5μg mL^{-1} Leupeptin) by vortexing and incubating in ice for 15 min. The cell homogenates were centrifuged three times transferring the supernatant into a new tube each time. Protein concentrations in the final clarified supernatants were measured (Lowry et al., 1951). Aliquots containing 20μg of total protein in 100μL total volume were added with 2, 4 and 6μL of spinach NDP kinase I antisera and incubated in ice for 1 h. Then 20μL of protein A-agarose (50% v/v in lysis buffer) was added into each tube and incubated at 4°C with rocking for 1 h. The agarose beads were pelleted by a 15 S pulse in the microfuge and the supernatant was discarded. The beads were washed three times with 1mL of lysis buffer each time. Finally, the proteins adhered onto agarose beads were released by heating to 85°C in 40 μL of 2X Laemmli buffer for 10 min. Equal volumes (10μL) of extract from each sample were separated on SDS-PAGE and labelled proteins were visualized by fluorography.

Partial purification of sugarcane and tobacco proteins for NDP kinase assay. Sugarcane and tobacco cells were incubated at 25°C or 38°C for 2 h, and 3g fresh weight of cells were harvested as described before. The cells were frozen and ground in liq. N_2 and extracted for 1 h with 30 mL 100% ice cold acetone.

The acetone extracted cells were filtered, further washed with 10 mL of acetone and vacuum dried. The acetone powder was extracted for 5 h into 6mL of ice cold buffer A (chapter III) with stirring, filtered through miracloth and centrifuged in SS34 rotor at 11,200 rpm for 20 min at 1°C. The supernatants were loaded separately onto DEAE-sepharose columns (5mL) by gravity. The flow through fractions were reloaded onto the columns and finally the columns were washed thoroughly with 30mL of buffer B (chapter III) containing 50mM NaCl. The columns were developed sequentially with 10 mL each of buffer B containing 0.1M, 0.2M, 0.4M and 0.8M NaCl. The fractions containing 16.5-18kD autophosphorylation activity were dialyzed exhaustively against 10mM ammonium bicarbonate and lyophilized. The lyophilized samples were resuspended in 100mL milli-Q H_2O, and total protein concentrations were measured according to Lowry's assay. Aliquots equivalent to 1μg of total protein from each sample were used to assay the NDP kinase activity. The NDP kinase assay was performed as described in chapter III.

RESULTS AND DISCUSSION

Autophosphorylation of NDP kinase in response to heat shock. Cultured sugarcane cells were exposed to 25 (control), 34, 36, 38, 40 and 42°C for 2 h, and total cellular proteins were extracted into 2X Laemmli buffer. Results of the *in situ* phosphorylation of proteins from each treatment are given in figure

11 A. Incorporation of labelled phosphate into NDP kinase increased approximately 4-fold in 42°C treated samples compared to control (figure 11). A minimum of 36°C heat shock was required to observe a change in phosphorylation level.

According to the previous work on the same cell line, the induction of low molecular weight HSPs requires a minimum of 36°C HS (Moisyadi and Harrington, 1989). The enhancement and decay of this autophosphorylation is parallel to the induction and decay of low molecular weight HSPs and thermotolerance (Moisyadi, 1991). Based on these observations, Moisyadi (1991) suggested that the low molecular weight autophosphorylating protein in cultured sugarcane cells may be HS induced or enhanced, however, the possibility that this autophosphorylation is due to increased activation of the protein could not be eliminated. The present data with cells treated at different HS temperatures indicated that increasing temperature had a direct correlation with the level of autophosphorylation of NDP kinase, the highest level being at the highest temperature tested (42°C) (figure 11). Interestingly, the increase in autophosphorylation of NDP kinase at 36°C (1.4-fold) in the present experiment was considerably less than that previously reported for the same cell line (3 to 4-fold) (Moisyadi et al., 1994). A 42°C HS was required to obtain a similar induction in the present experiment (figure 11 B). According to the

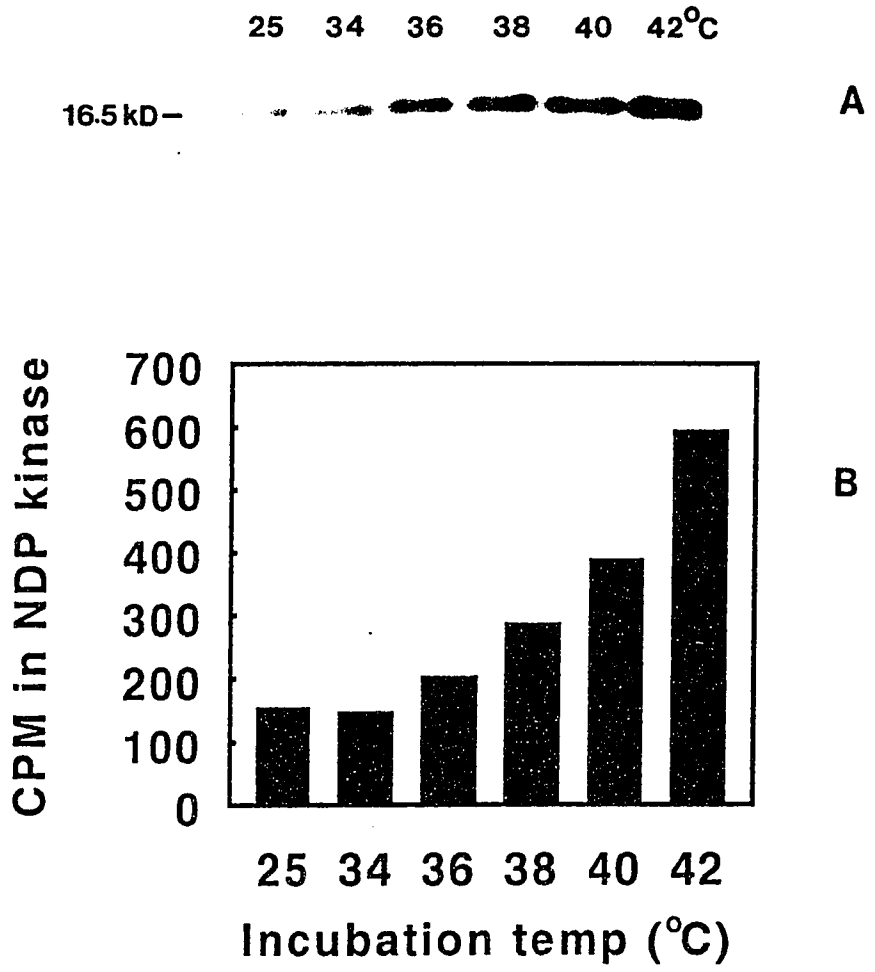

Figure 11. Effect of heat shock on the *in situ* phosphorylation activity of NDP kinase. Total protein extracts from sugarcane cells incubated at the indicated temperatures were separated by SDS-PAGE on gels containing 20μg mL^{-1} histone . Each lane contains 20μg protein. Panel A: Autoradiograph of gell *in situ* phosphorylated with ^{32}P-labeled ATP, Panel B : Analysis of 16-17 kD region of panel A gel by liquid scintillation spectrometry.

general characteristics of the HSR, approximately a 10°C above the normal growth temperature is required by many organisms to demonstrate HSR (Lindquist, 1986; Mansfield and Key, 1984). Therefore, the increased HS temperature required by sugarcane cells could be a direct consequence of the elevated growth temperature. The normal growth temperature of the sugarcane cells used in this laboratory was increased from 22-23°C to approximately 25-26°C during the lapse between the two experiments and may have contributed to the observed discrepancy. This observation further suggests that the phosphorylation activity of NDP kinase is a dynamic process regulated by the environmental conditions.

The changes in autophosphorylation of NDP kinase in cultured sugarcane cells in response to HS may be controlled by one or more of the following regulatory mechanisms; 1) the enhancement of the NDP kinase gene expression, 2) increased mRNA stability, 3) increased translation of the mRNA, 4) dephosphorylation of pre-existing protein or 5) changed configuration of the protein leading to increased ability to phosphorylate. The first 2 mechanisms involve changes in mRNA and protein level, while the 3rd possibility requires a steady state level of mRNA with an increased protein synthesis. The last 2 possibilities involve only the post-translational modifications of the protein. In

the subsequent data presented in this chapter, results of the experiments conducted to test some of these possibilities are reported.

Northern hybridization. Total and poly (A) RNAs were isolated from sugarcane cells treated for 2 h at 25 (control), 34, 36, 38, 40 and 42°C, and transferred onto nylon membrane after denaturing agarose gel electrophoresis. The RNAs on the membranes were hybridized with a labelled probe prepared from the cDNA insert pSCNDK8. The results of the northern blot analyses of total RNA and poly (A) RNA are shown in figure 12. The NDP kinase cDNA hybridized to a single band of 0.91 kb. The size of the mRNA matched the observed lengths of most cDNA inserts generated by cDNA library screening (0.62-0.78kb, see table 3). Considering the possible length of the poly A tail (approximately 0.1-0.2kb), the size of the mRNA indicated that at least few of the isolated cDNA clones were close to full native length.

The size of the sugarcane NDP kinase mRNA (0.91kb) is considerably smaller than that of spinach (1.1kb) (Nomura et al., 1992). In spinach, both NDP kinase I and II probes hybridized to mRNAs of similar size, although the open reading frames were considerably different; 444bp for NDP kinase I and 699bp for NDP kinase II (Nomura et al., 1992; Zhang et al., 1993). It is possible that in sugarcane, the NDP kinase II mRNA is similar in length to

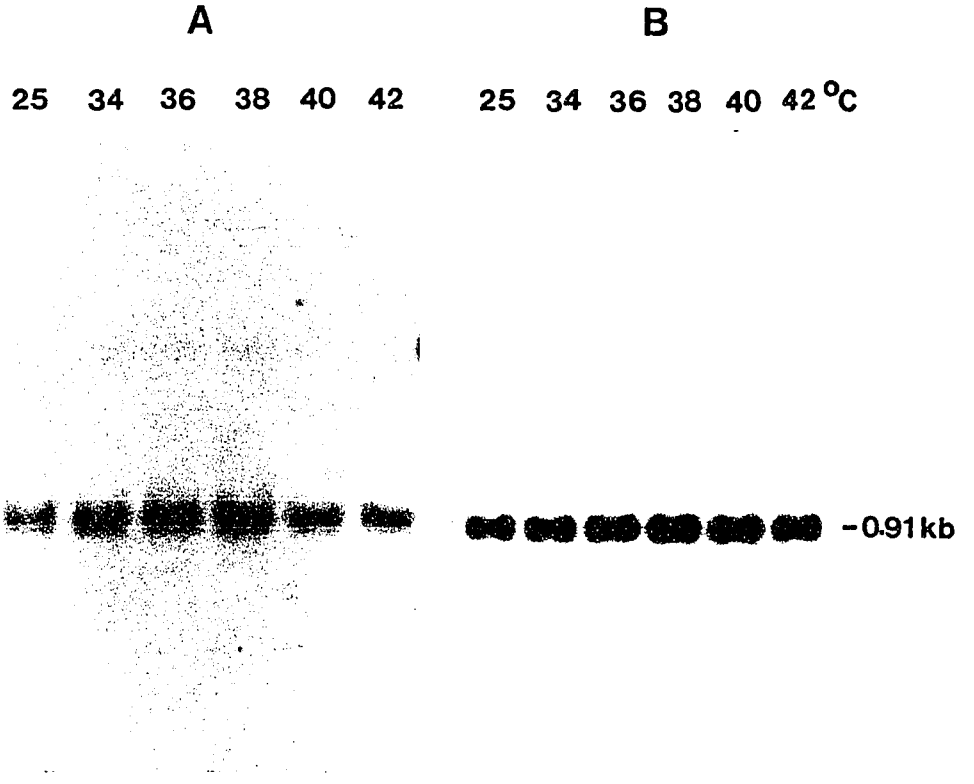

Figure 12. Northern hybridization of total and poly (A) RNA from sugarcane cells heat-shocked at different temperatures for 2 h. The RNAs were hybridized to pSCNDK8 cDNA probe. Panel A: each lane was loaded with 10μg total RNA, Panel B: each lane was loaded with 1μg poly (A) RNA.

NDP kinase I mRNA, or the abundance and homology of NDP kinase II mRNA is too low to be detected by hybridization with an NDP kinase I cDNA probe.

The results of the northern blot hybridization indicated that the mRNA level for NDP kinase I in cultured sugarcane cells did not change in response to different HS temperatures (figure 12). The slight increase of message level at 38 - 40°C with a decline at 42°C back to control levels did not match the observed changes in the *in situ* phosphorylation activity. Further, this change was not consistantly observed in repeated attempts. In contrast, the autophosphorylation of NDP kinase gradually increased up to approximately 3 to 4-fold with increased incubation temperature (figure 11). These observations suggest that post-transcriptional events may be involved in the synthesis and differential phosphorylation of NDP kinase in response to HS. However, the possibility that mRNA synthesis as well as turnover increased during HS, thus maintaining the same steady state level of mRNA in the cells, could not be eliminated based solely on the data presented here. A more detailed analysis employing nuclear run-on techniques should provide conclusive evidence in this aspect.

Western blot analysis of NDP kinase in sugarcane cells. Protein extracts obtained from cells treated at the above temperatures were analyzed for NDP kinase levels by direct immunoblotting (figure 13). The antibodies reacted strongly with a major polypeptide band at 16.5 kD with a weak signal at 18 kD. The results indicated that the levels of the NDP kinase protein did not change considerably in response to heat shock (figure 13). Thus, the increase in autophosphorylation as shown in figure 11 was not due to increased protein levels.

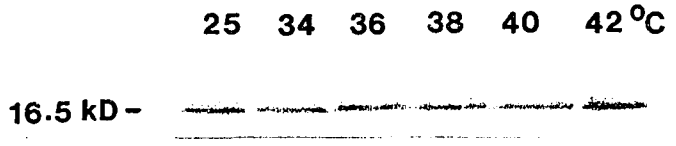

Figure 13. Effect of heat shock on sugarcane NDP kinase levels. Total proteins extracted from cells heat-shocked at different temperatures were separated by SDS-PAGE and immuno detected with anti-spinach NDPK I antibody. Each lane was loaded with 20μg total protein.

The observed very slight increase in NDP kinase level at 42°C may be due to increased synthesis. However, it is also possible that at 42°C, synthesis of NDP kinase is unaffected while the synthesis of many other normal proteins are reduced, thus over-representing NDP kinase in total protein extracts. According to the previous work in this cell line, 2 h heat treatment at 42°C and above substantially reduces the normal protein synthesis, with some reduction in HSP synthesis as well (Moisyadi and Harrington, 1989). Thus the credibility of the latter possibility cannot be excluded. However, the increased or persistant synthesis of NDP kinase at high temperatures also suggest the potential importance of this enzyme in heat-shocked cells.

Two dimensional IEF/SDS-PAGE and western analysis. Proteins extracted from cells treated at 25°C and 40°C were separated by two dimensional IEF/SDS-PAGE, transferred onto PVDF membrane and immunoreacted with spinach anti-NDP kinase I antibody (figure 14). In addition to 16.5-18kD polypeptides, many other polypeptides of varying molecular masses and isoelectric points reacted with the spinach NDP kinase I antibodies. This may be explained by the polyclonal nature of the antibodies and the presence of several conserved domains that cross react with kinases and nucleotide binding proteins.

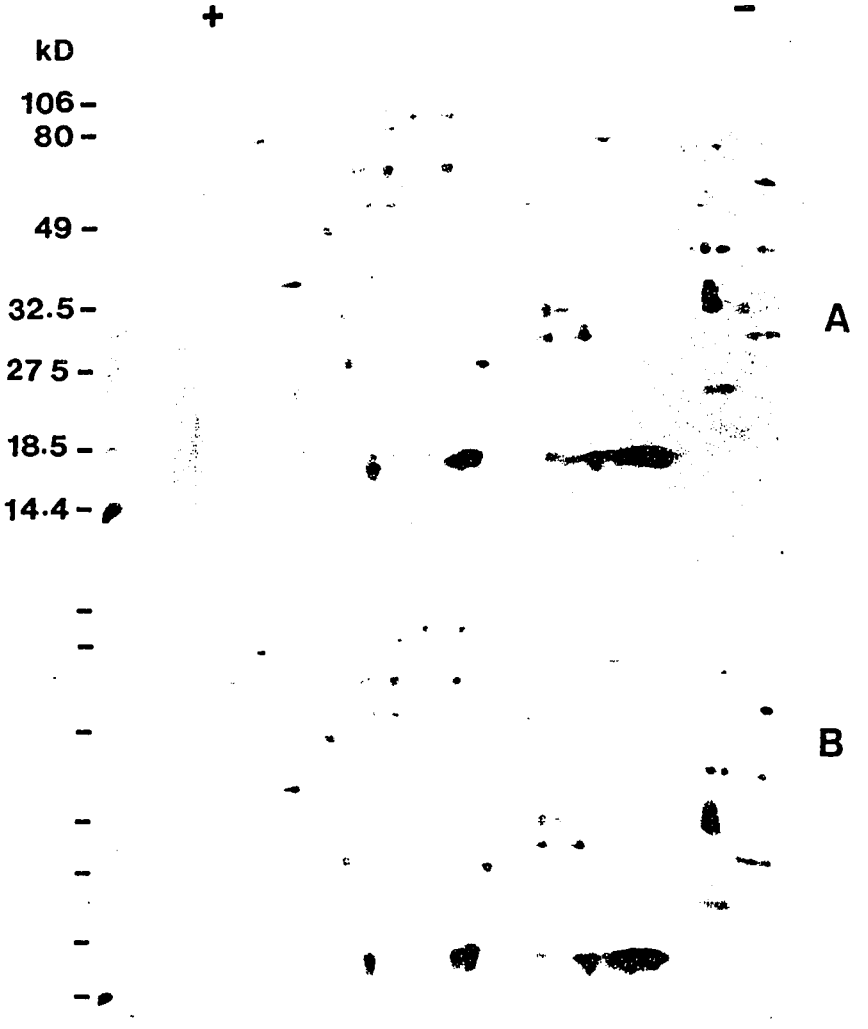

Figure 14. Immuno detection of sugarcane NDP kinase after two dimensional IEF/SDS-PAGE. Total proteins extracted from sugarcane cells incubated at 25°C (control) and 40°C (HS) for 2 h were separated on 2-dimensional gels and detected with anti spinach NDP kinase I antibody. Each gel was loaded with 40μg total protein. Panel A, 25°C control; Panel B, 2 h HS at 40°C

No major qualitative and quantitative changes in the levels of NDP kinase II isoforms were detected. However, two isoforms in the 16.5 kD size class showed a slightly increased intensity in HS sample (figure 14 B, arrows). If this change was due to increased synthesis or changed pattern of migration due to a post-translational modification (i.e. phosphorylation) during HS was not clear.

Effect of culture age on NDP kinase syntheis and phosphorylation. Proteins extracted from cells harvested (with or without a 2 h HS at 40°C) at different culture ages were separated by SDS-PAGE and either subjected to *in situ* phosphorylation with γ-^{32}P-ATP (phosphorylation method 1, Moisyadi et al., 1994), or immunoblotted. The autophosphorylation was high in cells freshly transferred into new culture media, and the enhancement of phosphorylation due to HS was also considerably high in these cells (figure 15). In cells from cultures older than 21 days, the autophosphorylation was not enhanced by HS.

Western blot analysis revealed a high level of NDP kinase in cells in fresh culture, with a gradual reduction with increasing culture age (figure 16 A and B). Approximately 24 days after transferring into new culture, the NDP kinase in these cells declined (figure 16 A). A HS at 40°C did not visibly

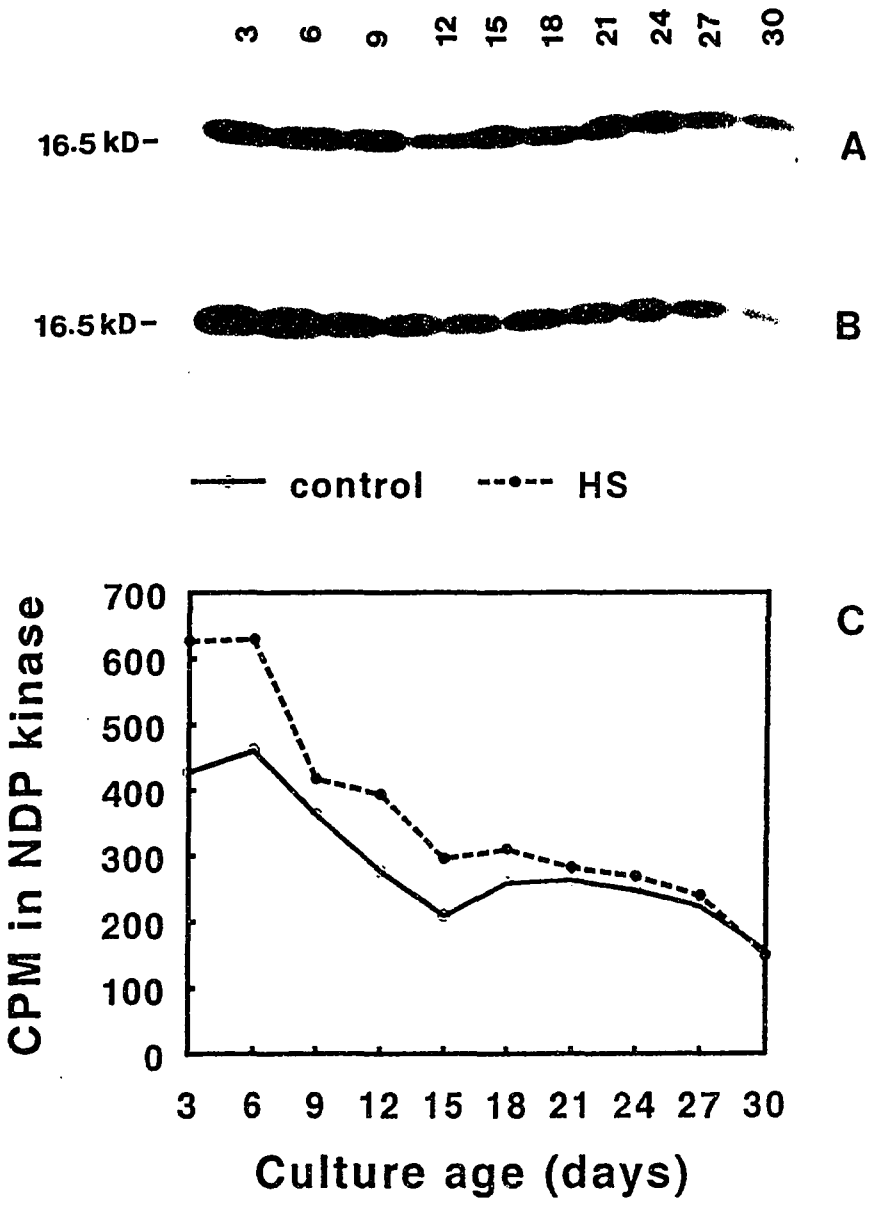

Figure 15. *In situ* phosphorylation of sugarcane NDP kinase at different culture ages. Each lane was loaded with 20μg of total proteins. Panel A : control, Panel B: after HS at 40°C for 2 h, Panel C: incorporation of ^{32}P into NDP kinase in panel A and B gels.

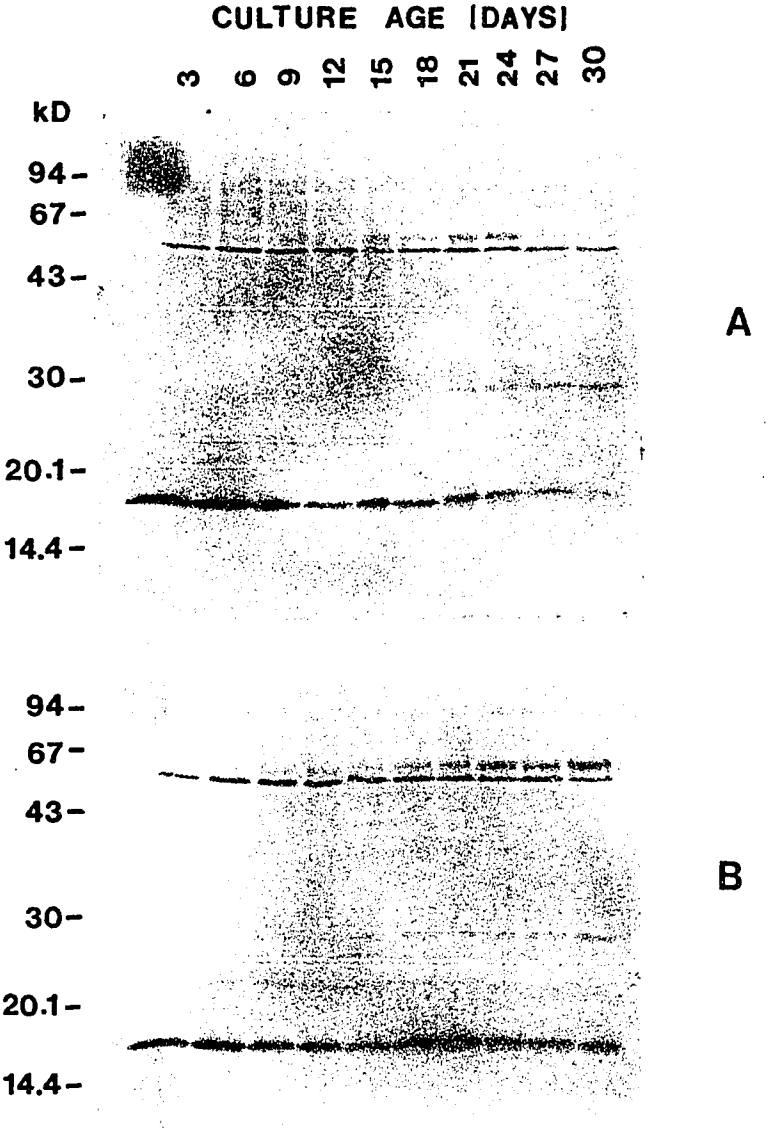

Figure 16. Immuno detection of NDP kinase in sugarcane cells at different culture ages. Each lane was loaded with 20μg of total proteins. Panel A: control, Panel B: after 2 h HS at 40°C.

increase the NDP kinase level in cells in fresh culture medium, however, showed a detectable increase in cultures older than 24 days (figure 16 B). This suggests that the synthesis of NDP kinase may be developmentally and environmentally regulated. Apparently, when the NDP kinase levels were high due to developmental regulation, HS did not further increase the levels, however, altered the ability of the already synthesized protein to autophosphorylate . In contrast, when the NDP kinase levels were low (i.e. in older cell cultures), HS increased the synthesis but not the phosphorylation (figure 16 A and B). These observations further suggest that synthesis and phosphorylation of NDP kinase are differentially regulated by HS.

The reason for the presence of high NDP kinase levels in cells transferred into fresh media is currently not clear. A correlation between NDP kinase activity and growth rate or development has been shown in several systems including plants and animals (Dickinson and Davies, 1971; Berges and Harrison, 1993). Considering the enzymatic role of NDP kinase to generate nonadenine nucleotides in the cells, especially GTP, this observation appears to be highly justifiable. Rapid growth and development involve the energy requiring processes including the synthesis of DNA, RNA, protein and fatty acids which demand increased synthesis of nonadenine nucleotides (Parks and Agarwal, 1973). In such cases, an increased activity of NDP kinase is also

required. Additionally, NDP kinase is also involved in various other cellular processes requiring GTP as the energy source such as G-protein activity and cell motility (see chapter I).

According to the results presented in figure 15 and 16, the synthesis of NDP kinase is enhanced when cultured sugarcane cells at mid-log phase are transferred into new culture media. If this relationship is a direct consequence of the high demand for nonadenine nucleotides in rapidly growing and dividing cells, or a response to changed culture conditions is not clear. Apparently, the higher level of *in situ* phosphorylation observed in the NDP kinase from young cell culture (figure 15 A) was due to the increased level of the protein itself (figure 16 A). However, the HS enhancement of phosphorylation status of NDP kinase from young cell culture, but not from the old cell culture (figure 15 B) indicated that environmentally regulated kinase activity was more prominent in actively growing cells. The enhanced *in situ* phosphorylation observed in response to HS in young culture cells could be due to two reasons; 1) in young and active cells, NDP kinase configuration changes during HS providing more phosphorylatable sites (i.e serine residues), and 2) in young cells HS activates or induces a phosphatase that dephosphorylates NDP kinase providing more sites for *in situ* phosphorylation. The credibility of these

possibilities require an in depth analysis of the actual phosphorylation state of the protein *in vivo* in response to cell age and HS.

In vivo ^{35}S labelling and immunoprecipitation of NDP kinase. Cultured sugarcane cells were *in vivo* labelled with Tran-^{35}S-label, proteins were extracted and immunoprecipitated with spinach anti-NDP kinase I antibody. The antibodies precipitated a number of labelled proteins of varying molecular masses (figure 17 A); however, most of these proteins appeared to be non-specifically precipitated, since a subsequent direct immunoblotting of the immunoprecipitated proteins with the same antibody resulted in fewer bands including a 16.5 kD band and 5 other major bands at 48.5, 39.7, 32.5, 28.7 and 20.6 kD (figure 17 B). It is possible that the 48.5 and 32.5 kD bands represent a trimer and a less abundant dimer of the 16.5 kD protein. In immunoblots of total cell extracts these bands were either absent or represented as minor bands. The conditions used for the extraction of labelled proteins for immunoprecipitation may have favored trimerization of the protein, which may not have completely dissociated by heating to 80°C in 2X Laemmli buffer during final extraction. Moisyadi (1991) reported that purified pp18 separated into several bands, i.e the polymers of the purified protein monomer, during SDS-PAGE. The appearance of these different polymers was due to

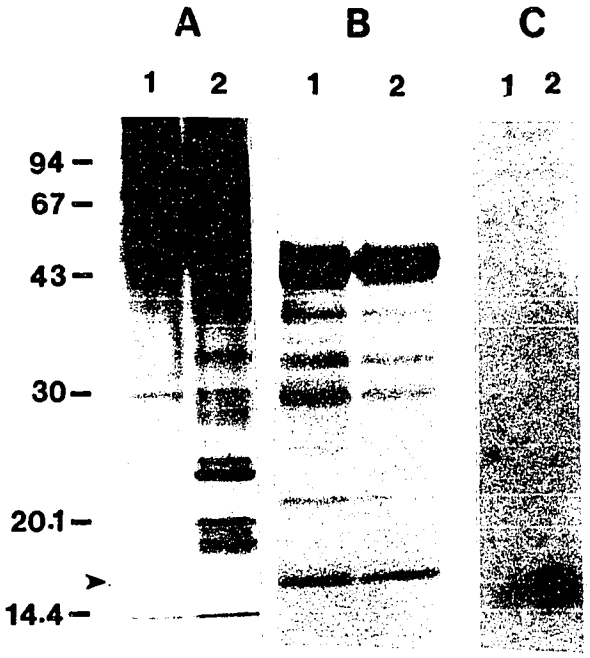

Figure 17. Immunoprecipitation of NDP kinase from *in vivo* ^{35}S-labelled sugarcane cell extracts. Panel A: Fluorograph of immunoprecipitated ^{35}S-labeled proteins, Panel B: immunoblotting of precipitated proteins with anti spinach NDPK I antibody, Panel C: *in situ* phosphorylation of precipitated proteins. Lanes 1, control (25°C); 2, HS for 2 h at 40°C.

electrophoresis conditions and the presence of different reducing agents in the extraction buffer (Moisyadi, 1991)

Presence of various contaminating bands in the immunoprecipitated NDP kinase preparation could also be due to the polyclonal nature of the spinach NDP kinase I antibody (Nomura et al., 1991). Nucleoside diphosphate kinase protein consists of several highly conserved regions including nucleotide binding domain and kinase domain (discussion in chapter IV) which are likely to recognize many other functionally related proteins.

According to the figure 17, *de novo* synthesis of NDP kinase was not enhanced in response to a 2 h HS at 40°C, but rather remained unaffected. In fact, the slight reduction observed in label incorporation into NDP kinase during HS as seen in figure 17 may have caused by manipulation errors during protein estimations or gel loading, since the background intensity of the lane corresponding to the HS sample was lower. However, when these immunoprecipitated proteins were *in situ* phosphorylated, the 16.5 kD band in the HS sample showed a considerably higher intensity over the control sample (figure 17 C), further supporting the observation that NDP kinase undergoes changes in autophosphorylation activity during HS.

NDP kinase activities during HS. When the partially purified sugarcane protein extracts were analyzed for the NDP kinase activity, the 40°C HS sample consistently showed 1.5- to 2-fold higher activity over the 25°C control sample (figure 18 A). However, as discussed before in this chapter, the NDP kinase protein synthesis did not undergo major changes during 40°C HS (see figure 17). Thus, this increased NDP kinase enzyme activity in HS cells was most probably due to post-translational activation of the enzyme during HS. If the observed changes in phosphorylation status had a functional relationship with the increase in NDP kinase activity during HS needs further investigation. Interestingly, when tobacco cells treated for 3 h at 25, 34, 36, 38, 40 and 42°C, the autophosphorylation of NDP kinase was unaffected (figure 19). This was in contrast to the sugarcane cells which showed approximately a 3 to 4-fold increase in *in situ* autophosphorylation activity in HS samples (see figure 11). Additionally, the NDP kinase activity in protein extracts from heat shocked tobacco cells was not increased in response to HS (figure 18).

Cultured sugarcane is among the few systems in which the normal protein synthesis is not suppressed at sublethal HS treatments which induce a full spectrum of HSPs (Moisyadi and Harrington, 1989). This suggests that in

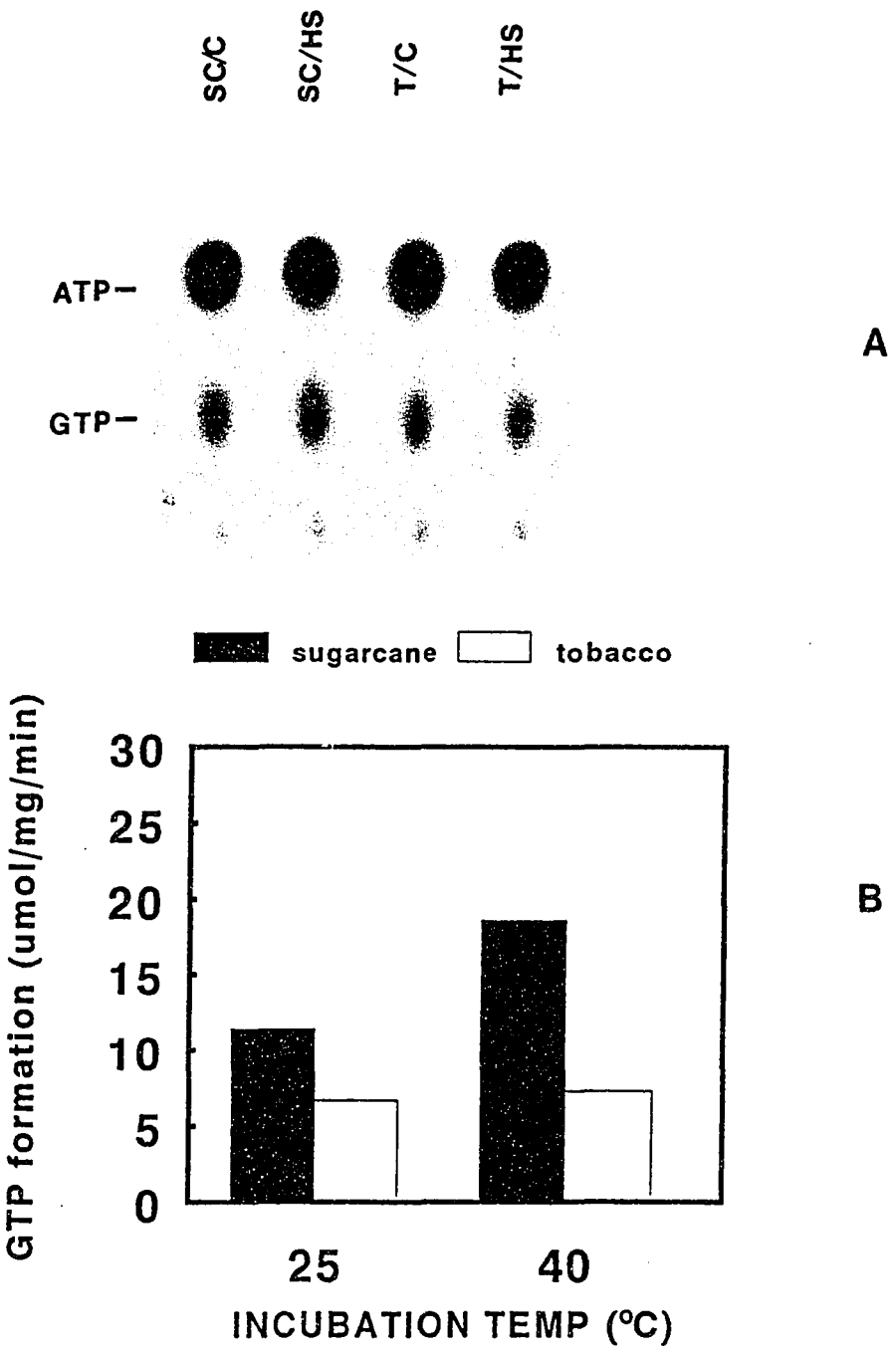

Figure 18. NDP kinase activities of sugarcane and tobacco cells in response to HS at 40°C for 2 h. Panel A: autoradiograph of TLC showing labeled GTP formation, Panel B: rates of GTP formation by each sample.

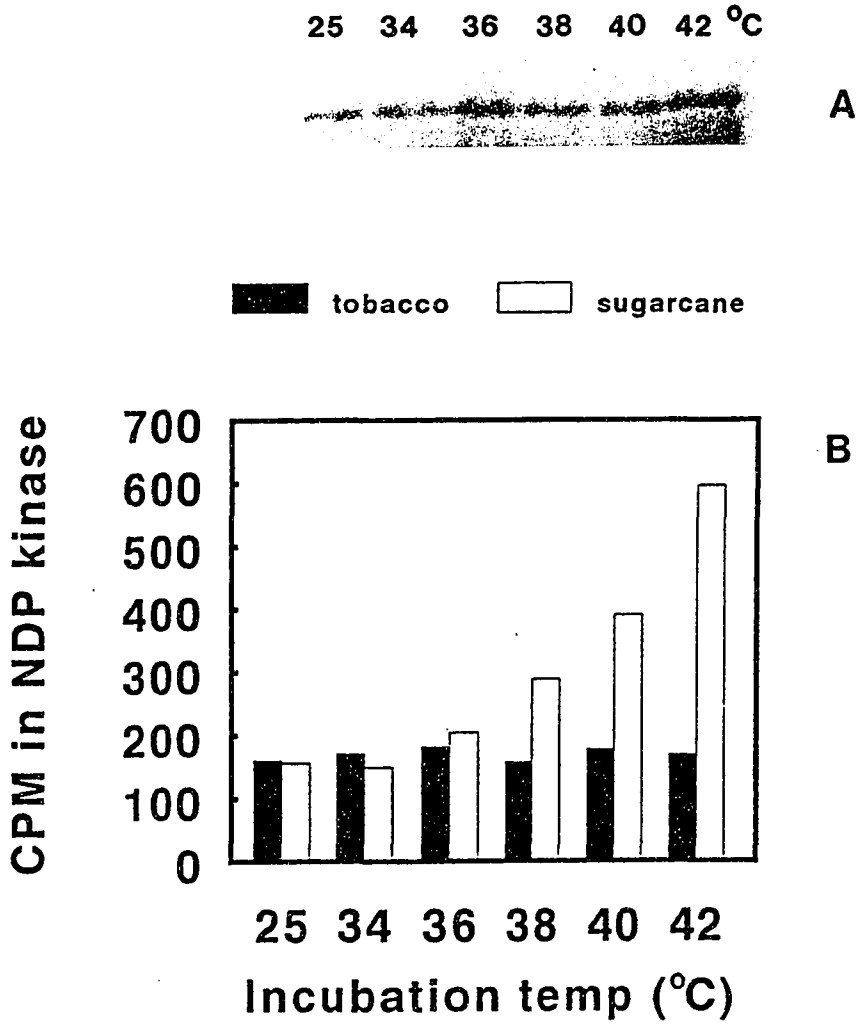

Figure 19. Effect of heat shock on autophosphorylation activity of tobacco NDP kinase. Panel A: autoradiograph of *in situ* phosphorylated tobacco proteins. Each lane was loaded with 20μg of total proteins, Panel B: analysis of label incorporation into NDP kinase

this system protein synthesis machinery is not affected by HS treatments. If the present observation that the enhanced activity of NDP kinase had a functional relationship to the rescue of constitutive protein synthesis in sugarcane cells during HS deserves further investigation. In fact, GTP to GDP ratio is a determining factor for the formation of initiation complex (Walton and Gil, 1975). One of the major roles of NDP kinases is to maintain the nonadenine nucleotide (GTP, CTP, UTP and TTP) concentrations in the cell (Parks and Agarwal, 1973), thus may be an important regulator of protein synthesis. This idea was further supported, although indirectly, by the observation that in tobacco cells NDP kinase enzyme activity or phosphorylation status did not change during HS (figures 18 and 19). Previous reports show that in tobacco cells, the constitutive protein synthesis is reduced dramatically during HS (Kanabus et al., 1986; Harrington and Alm, 1988, Dharmasiri, 1990).

NDP kinase levels in intact sugarcane shoots. In contrast to the cultured sugarcane cells, intact sugarcane shoots showed a different pattern of mRNA levels in response to HS. In this study, young (4d old) sugarcane shoots were subjected to 40°C HS for different times, total RNAs were extracted and hybridized to sugarcane NDP kinase cDNA clone pSCNDK8 (figure 20). The NDP kinase transcript level was increased substantially during a 2 h HS and declined after HS for 5 h (figure 20, lanes 4 and 5). In a recent report on

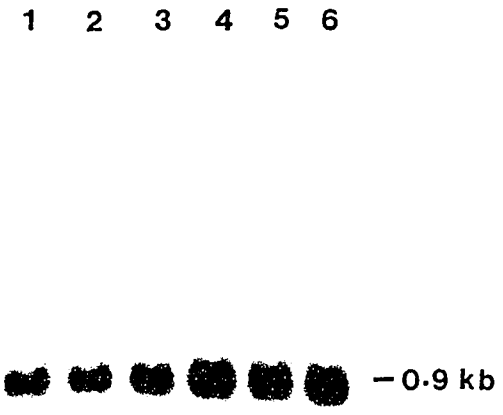

Figure 20. Effect of heat shock (40°C) on the total RNA levels in sugarcane shoots. Each lane was loaded with 10μg of total RNA and probed with pSCNDK8 cDNA. Lane 1, control; 2, 30 min HS; 3, 1 h HS; 4, 2 h HS; 5, 5 h HS; 6, 5 h after wounding

tomato plants, NDP kinase transcript levels were shown to be elevated within 1 h of wounding, reaching a plateau after 6 h (Harris et al., 1994). Interestingly, in sugarcane shoots, the 2 h HS and a 5 h wounding resulted in similar levels of NDP kinase transcript (figure 20, lanes 4 and 6), suggesting that the NDP kinase gene promoter may possess elements that respond to environmental stresses. Why cultured sugarcane cells did not respond similar to intact shoots is an interesting dilemma. In cultured sugarcane cells NDP kinase transcript level may be enhanced to a high level, so that a further enhancement could not be detected by providing a HS treatment.

CHAPTER VI

CONCLUSIONS AND FUTURE PROSPECTS

A low molecular mass autophosphorylating protein (pp18) previously characterized in cultured sugarcane cells and pea bud plasma membranes as a catalytic subunit of a unique protein kinase was identified as an NDP kinase. All the characteristics reported for the pp18 in pea and sugarcane could be explained by the general features of NDP kinases from different sources. For example, molecular masses, presence of multiple isoforms, the ability of pp18 to autophosphorylate on serine residues, the rapid turnover of the phosphorylation signal and the weak protein kinase activity toward nonspecific substrates such as histone have all been previously reported for NDP kinases.

The nucleotide sequences of four cDNAs encoding sugarcane NDP kinase I showed a very high identity to each other. Deduced amino acid sequences of three of these clones (pSCNDK 8, 10 and 19) also showed a remarkable homology with previously reported NDP kinase sequences from various organisms. These 3 sugarcane sequences contained all the conserved features characterized in NDP kinase primary structures such as the nucleotide binding domain, phosphorylatable histidine residue which acts as the phosphoenzyme intermediate, the phosphate transfer domain which is

responsible for kinase function, and also a putative serine residue that may be autophosphorylated. In contrast, pSCNDK5 cDNA showed a frame shift after the codon for the 97th amino acid residue, thus resulting in a 55 amino acid stretch with no homology to NDP kinase. If this modified NDP kinase protein is expressed *in vivo* in sugarcane cells or due to a cloning artifact is currently not clear. Western blots of total sugarcane proteins with monoclonal antibodies raised against a synthetic peptide based on the modified portion of the protein should provide an answer to this question. A carboxyl terminal peptide fragment from the purified expression SCNDK5 protein from λgt11 may also be used for raising antibodies. If there is a functional significance of this mutation is currently unknown. Despite the differences at protein level, the very high identity between the four cDNA sequences indicate that they may belong to a highly conserved gene family.

The sugarcane cell extracts exhibited increased autophosphorylation and NDP kinase activity in response to HS. This increase was not paralleled by increased mRNA levels, however, a slight increase in protein synthesis was detected especially at high incubation temperatures. Thus, both post-transcriptional regulation of mRNAs and post-translational modifications of the protein may have contributed to the observed enhancement in phosphorylation.

The age of the cell culture also plays an important role in the synthesis and phosphorylation of NDP kinase in response to HS.

In contrast to cultured cells, intact sugarcane shoots showed an elevated NDP kinase mRNA level in response to a 2 h HS. A similar level of enhancement was observed in sugarcane shoots 5 h after wounding, suggesting that sugarcane NDP kinase gene may have stress response elements.

Future prospects. The results presented in this study supported the idea that NDP kinase in cultured sugarcane cells exists as multiple isoforms. Separation to homogeneity and biochemical characterizations of two major classes of isoforms (16.5 and 18 kD) should facilitate an understanding of the enzymatic roles of these to types of isoforms.

The NDP kinase cDNAs sequenced in this study showed minor differences at the nucleotide level suggesting the need of a thorough analysis of the cDNA library for clones representing different isoforms. Search of the possible functional differences of the protein encoded by pSCNDK5 may further facilitate the understanding of multiple roles of NDP kinase. Deducing the primary structures will facilitate the characterization of various isoforms possibly localized in different subcellular compartments (i.e. the presence of

signal peptides). Kinetic assays of purified isoforms should also provide an understanding of their putative functions. These assays may also be performed on purified expression proteins.

How NDP kinase activity in cultured sugarcane cells is regulated during HS still remains as an unanswered question. If altered phosphorylation status is a factor, more direct approaches such as metabolic labelling of cells with labelled phosphate followed by analysis of the changes in phosphoamino acids should provide a better picture. Generation of NDP kinase specific monoclonal antibodies based on the deduced amino acid sequences may be highly desirable for immunoprecipitation studies. More direct approaches such as radio-immunological assays may also provide a more accurate picture of the changes in NDP kinase synthesis during HS. The possibility that NDP kinase is a factor in differential regulation of protein synthesis machinery in sugarcane and tobacco cells during HS appears to be an interesting and important area to be investigated. Finally, the apparent HS enhancement of NDP kinase transcript levels in sugarcane shoots indicated that promoter sequences of NDP kinase genes may contain stress responsive elements. Isolation of the sugarcane genes encoding different isoforms and characterizing their promoter regions appear to be highly promising.

APPENDIX

1. a) Prehybridization/hybridization solution for Southern blots.

   5X Denhardt's solution

   6X SSPE

   0.5% SDS

   50μg mL^{-1} Salmon sperm DNA (denatured)

   b) Washing solutions and conditions for Southern blots.

   i) 5X SSC, 0.5% SDS at room temperature, 2 X 15 min

   ii) 1X SSC, 1% SDS at 37°C, 2 X 15 min

   iii) 0.1X SSC, 1% SDS at 65°C, 2 X 15 min or as necessary

2. a) Prehybridization/hybridization solution for Northern blots.

   50% formamide

   5X Denhardt's solution

   5X SSPE

   0.2% SDS

   75μg mL^{-1} Salmon sperm DNA (denatured)

   b) Washing solutions and conditions for Northern blots

   i) 5X SSPE, 0.5% SDS at room temperature, 2 X 15 min

   ii) 1X SSPE, 1% SDS at 37°C, 2 X 15 min

   iii) 0.1X SSPE, 1% SDS at 65°C, 2 X 15 min or as necessary

(Ref: Manufacturer's Instructions, MSI nylon membranes)

3. Blocking and washing reageats for Western blot analysis.

(Dr. Samuel S.M. Sun, Department of Plant Molecular Physiology, University of Hawaii, personal communications)

a) Blocking reagent (Blotto)

5% non fat dry milk

0.01% Antifoam A emulsion

150mM NaCl

15mM Na_2HPO_4 (dibasic)

4mM NaH_2PO_4 (monobasic)

pH 7.2 with HCl

b) Washing solutions and procedure

i) Block the membrane in blotto, 1 hr

wash 2 X 10min in TTBS (0.1% Tween-20 in 20mM Tris, 0.5M NaCl, pH7.2)

ii) Primary antibody solution (rabbit anti-spinach NDP kinase I), 1-2 hr

iii) Rinse 2X, wash 2 X 5 min and 1 X 15 min in TTBS

iv) Second antibody solution (goat anti-rabbit alkaline phosphatase conjugated), 1-2 hr

v) Rinse 2X, wash 2 X 5 min, 1 X 15 min in TTBS

vi) Wash 2 X 5 min, 1 X 15 min in TBS (20mM Tris, O.5M NaCl, pH 7.2)

vii) Color development with BCIP and Nitro BT

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