Novel Functions of the Mitochondrial Nucleoside Diphosphate Kinase in Plants

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Front cover illustration: Fluorescent images showing an *Arabidopsis thaliana* protoplast. From left to right: chloroplast, NDPK-GFP and mitotracker fluorescences.

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"Reality leaves a lot to the imagination."

J. Lennon

Abstract

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Nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes found in almost all species. Their main function is to transfer a γ-phosphate from ATP to a cognate nucleoside diphosphate, thereby balancing the nucleoside pool. Early studies showed that the ablation of NDPK genes had different effects depending on species and isoform, indicating a multi-functionality of the NDPK enzymes. Indeed, additional functions, including nuclease and protein kinase activities, have been demonstrated and are continuously being discovered. Attention was especially paid to these additional effects when it was shown that a human NDPK (nm23) is involved in the progression of cancer.

In plants there are three NDPK isoforms. NDPK1 is located in the cytosol, NDPK2 in the chloroplast stroma and NDPK3 in the mitochondrial inner membrane and intermembrane space (IMS). The plant NDPKs have been implicated in stress and hormone response, as well as in light signalling. This work concerns the plant NDPK genes and proteins, with a specific focus on the mitochondrial NDPK3.

Phylogenetic analyses revealed that the evolution of separate plant NDPK isoforms is evolutionary old. In *Arabidopsis thaliana* the *NDPK3* gene has been duplicated relatively recently, resulting in two genes, *NDPK3a* and *NDPK3b*. Quantitative RT-PCR showed that the expression of the NDPK genes differs between tissues, with *NDPK1* being the overall most abundant transcript. The expression of the *NDPK3a* gene is relatively high in all tissues, especially in rapidly dividing cells in the flower bud. In addition, this gene appears to respond to the cell energy level, as shown by the more than 2-fold induction after sucrose and glucose applications. Interestingly, a specific expression of the *NDPK3b* gene was seen in ovules and tapetum cells of stage 12 flower buds, suggesting a specific function of this protein in these tissues despite a very low overall expression.

By GFP-fusions and immunoblotting we demonstrated that the pea NDPK3 is dually targeted to chloroplasts and mitochondria. In addition, we found that recombinant NDPK3 is able to interact with the mitochondrial Adenylate kinase 1 (AK1). Activity measurements showed an inhibition of AK1 activity in parallel to an augmented NDPK3 activity when both recombinant enzymes were present in the same assay. We were also able to show that the recombinant NDPK3 protein can cleave DNA and RNA molecules, an ability that was independent of the capacity to form a hexameric structure of the protein, and which was inhibited by ATP. This is the first time a nuclease has been identified in the plant IMS.

Keywords: energy, NDPK, ATP, IMS, Adenylate kinase, nuclease, tRNA, sucrose, glucose, WRKY.

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Sammanfattning

Alla levande varelser behöver energi för att överleva. Mitokondrien är en självständig del inuti cellen som omvandlar näring till den energivaluta som cellen använder, kallad ATP (adenosin-trifosfat). När ATPt är förbukat har det förlorat en fosfatgrupp, och bildat ADP (adenosin-difosfat). ATP används också vid uppbyggnad av cellens arvsmassa (DNA). Det finns tre molekyler till som används i DNA: GTP (guanosin-trifosfat), CTP (cytosin-trifosfat) och TTP (tymidin-trifosfat). Extra ATP, GTP, CTP och TTP kan behövas när nytt DNA ska byggas, till exempel vid celldelning. Det finns enzymer som omvandlar ATP-molekylen till GTP, CTP och TTP. Ett sådant enzym är Nukleosid-difosfat kinas (NDPK). Detta sker genom att NDPK överför en fosfatgrupp från ATP till de de övriga strukturerna när dessa har förlorat en fosfatgrupp, vilket inträffar vid olika reaktioner i cellen.

I denna avhandling har jag fokuserat på det mitokondriella NDPK-enzymet, NDPK3, även om jag också berör de två andra växt-NDPK-enzymerna, NDPK1 och NDPK2. Genom att studera fluorescerande proteiner har vi visat att NDPK3 inte bara finns i mitokondrien, utan också i kloroplasten (den del i cellen där fotosyntesen sker). Dessutom har vi funnit att NDPK3 reglerar funktionen av ett annat enzym som hanterar ATP, Adenylat kinas, och att NDPK3 kan klyva DNA. Detta kan betyda att NDPK3 är ett enzym som reparerar DNA, eller att det hjälper till att bryta ner "gammalt" DNA när en cell dör. Det är mycket betydelsefullt att detta fungerar optimalt, speciellt i människan, eftersom ett felaktigt "döende-förlopp" kan leda till cancer. I växter har samma process en viktig funktion i försvaret mot bakterier och svampar. För att kunna skydda grödor mot dessa skade-organismer är det viktigt att utreda hur denna process förlöper i växter.

Contents

Abbreviations

ADP	adenosine diphosphate
AK	adenylate kinase
AMP	adenosine monophosphate
ANT	adenine nucleotide translocator
ATP	adenosine triphsphate
CK	creatine kinase
CMS	cytoplasmic male sterility
CSP41	chloroplast stem loop-binding protein 41
Cyt *c*	cytochrome *c*
EDTA	ethylenediaminetetraacetic acid
Endo G	endonuclease G
FADH	flavin adenine dinucleotide
GFP	green fluorescent protein
HXK	hexokinase
IMS	intermembrane space
LhcbII	light harvesting complex IIb
MPP	mitochondrial processing peptidase
MSF	mitochondrial import stimulating factor
NADH	nicotinamide adenine dinucleotide
NDPK	nucleoside diphophate kinase
NHE	nuclease hypersensitive element
PBF	presequence binding factor
PCD	programmed cell death
PDGF	platelet derived growth factor
PDH	pyruvate dehydrogenase
PUMP	plant uncoupling protein
TCA	tricyclic acid
TIM	translocator of the inner membrane
TOM	translocator of the outer membrane
UCP	uncoupling protein
UTR	untranslated region
VDAC	voltage dependent anion channel

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Hammargren, J., Sundström, J., Johansson, M., Bergman, P. & Knorpp, C. 2007. On the phylogeny, expression and targeting of plant Nucleoside Diphosphate Kinases. *Physiologia Plantarum 129*, 79-89.

II. Hammargren, J., Salinas, T., Maréchal-Drouard, L. & Knorpp C. 2007. The pea mitochondrial Nucleoside Diphosphate Kinase cleaves DNA and RNA. *FEBS Letters 581*, 3507-3511.

III. Hammargren, J., Rosenquist, S., Jansson, C. & Knorpp, C. A novel connection between nucleotide and carbohydrate metabolism in mitochondria: The Arabidopsis Nucleoside Diphosphate Kinase 3a gene is regulated by sugars. (Manuscript).

IV. Johansson, M., Hammargren, J., Uppsäll E., MacKenzie A. & Knorpp C. The activities of Nucleoside Diphosphate Kinase and Adenylate Kinase are influenced by their interaction. (Manuscript).

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Related manuscript (not included in thesis):

Rosenquist, S., Hammargren, J., Knorpp, C. & Jansson, C. Arabidopsis WRKY Mutants Impaired in Sugar Signaling. (Manuscript).

Introduction

Mitochondria are multi-functional cellular compartments involved in a variety of processes. Their main function is to supply energy to the cell in the form of ATP created by oxidative phosphorylation. In addition, mitochondria are involved in the production of vitamins (Rebeille *et al.*, 2007), organic- and amino acids. Implications for this organelle in stress defence and detoxification have also been demonstrated (Noctor, De Paepe & Foyer, 2007; Salvi *et al.*, 2007). Mitochondrial defects cause severe diseases in mammals (Finsterer, 2006). Other energy-demanding processes, such as eye sight and hearing, are also often affected upon a compromised mitochondrial function (Fattal *et al.*, 2006; Johns, 1995).

In plants, the chloroplast compartment contributes to the ATP production. However, in non-photosynthetic tissue (*e.g.* roots and developing buds), and in the dark, mitochondrial oxidative phosphorylation is the only source of cellular ATP. Also in plants mitochondrial disturbances inflict problems. Disrupted intra-cellular communication between nucleus and mitochondria can result in cytoplasmic male sterility (CMS). However, the CMS trait has been extensively used to increase genetic variability of the plants and thereby augment yields (Chase, 2007).

Enzymes involved in nucleotide conversion are tightly linked to the energy metabolism of the cell. One group of such enzymes is the Nucleoside diphosphate kinases (NDPKs). NDPKs are proteins present in more or less all species as well as in most sub-cellular compartments, including mitochondria. It was long believed that the function of these enzymes was purely "house-keeping" in the equilibration of the nucleoside pool. However, lately additional intriguing tasks for these proteins have been discovered with implications in cancer progression and signal transduction. (Chakrabarty, 1998; Choi, Yi & Lee, 1999; Fan *et al.*, 2003; Postel *et al.*, 1993; Ryu *et al.*, 2005; Steeg *et al.*, 1988; Zimmermann *et al.*, 1999)

This thesis mainly concerns the plant mitochondrial NDPKs, focusing on the exploration of potential "new" NDPK functions.

Mitochondria

Origin

The endosymbiont theory is the most widely accepted hypothesis describing the origin of modern-day mitochondria. It states that a free-living bacterium once became incorporated into an ancient "proto-eukaryote", which evolved into the mitochondrion of today (Gray, 1999). Comparative studies have identified α-proteobacteria of the order *Rickettsiale* as the closest bacterial now-living mitochondrial relatives (Andersson *et al.*, 1996; Andersson *et al.*, 1998; Sicheritz-Ponten, Kurland & Andersson, 1998; Viale *et al.*, 1994). These are obligatory intracellular organisms, which cause diseases both in humans and in plants (Chen,

Campbell & Purcell, 1996; Davis *et al.*, 1998). The genome of the thyphoid fever-causing *Rickettsi prowazekii* was sequenced in 1998 (Andersson*, et al.*, 1998). Interestingly, some mitochondrial genes, *e.g.* those involved in transcription and replication, are not similar to those of *R. prowazekii*. Instead they resemble viral genes. It is believed that these virus-like genes are acquired from cryptic prophages present in the early endosymbiont (Filee & Forterre, 2005). This finding indicates that the "true" origin of the mitochondrion might be more complex than the simple addition of a bacterium to an ancient host cell.

Structure

Mitochondria were long thought to be discrete subcellular compartments. However, this notion was challenged when mitochondria were observed in the microscope in the 1950's, and it was proposed that they could form inter-mitochondrial interactions. The development of *in vivo* fluorescent labelling in combination with more advanced microscopy techniques has lately made it possible to demonstrate mitochondrial networks, which are constantly fusing and dividing (Bereiter-Hahn & Voth, 1994; Hoffmann & Avers, 1973; Nunnari *et al.*, 1997). For simplicity, the structure of a single mitochondrial entity is described here.

Mitochondria consist of two membranes (Fig. 1), out of which the outer is relatively permeable, permitting the transport of molecules of less than 2 kDa in size. Porins, also called Voltage-dependent anion channels (VDAC), mediate this free diffusion of metabolites (Colombini, 1979). VDACs are however not completely indiscriminatory, as different conformations of these proteins have demonstrated that they are anion or cation selective (Colombini, 1979). The inner membrane, on the other hand, is highly impermeable. Specific inner membrane transporters, *e.g.* the Adenine nucleotide translocator, also called ANT, ADP/ATP carrier protein, ADP/ATP translocase and Adenylate translocator, mediate the transfer of adenine nucleotides over this membrane. The respiratory chain is integrated into the inner membrane, the surface of which is highly folded in order to increase the area that can be used for respiration. The tricyclic acid (TCA) cycle, as well as the mitochondrial genome, is found in the matrix, which is enclosed by the inner membrane. Between the inner and the outer membrane is the intermembrane space (IMS). Proteins involved in nucleotide metabolism, such as NDPKs and Adenylate kinases (AK), are located in this compartment, which, at least in mammals, harbours proteins released during the process of programmed cell death (PCD, Bras, Queenan & Susin, 2005).

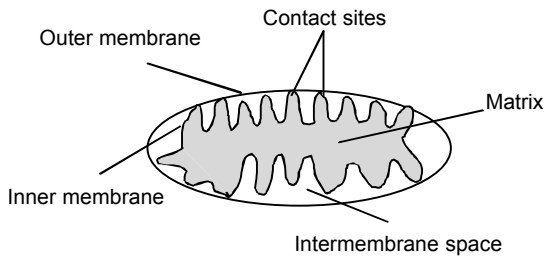

Fig 1. Schematic illustration of the mitochondrial compartments.

Mitochondrial protein interactions

Interestingly, many mitochondrial functions are carried out by enzymes forming multi-protein complexes. One example of this is the respiratory supercomplexes in the respiratory chain (Boekema & Braun, 2007; Eubel, Jansch & Braun, 2003), another is the protein complexes found between the inner and the outer mitochondrial membranes. It has been suggested that the assembly and disassembly of these sites are dynamic processes. In addition, recent studies show that supercomplexes involved in *e.g* transport and respiration are differently distributed within the inner membrane (Vogel *et al.*, 2006). The major part of the respiratory complexes is in the cristae part (the invaginations in the inner membrane), while the protein transport complexes are located in the inner boundary membrane (which are in closer contact with the outer membrane). Intriguingly, the location of the complexes can also change in response to cell status (Vogel*, et al.*, 2006).

In animal cells, an association between the VDAC and the ANT proteins has been extensively studied, and it was demonstrated that these proteins interact both directly and indirectly through Creatine kinase (CK, Beutner *et al.*, 1996). Interestingly, the direct interaction between VDAC and ANT forces VDAC to adapt a conformation with high affinity for a kinase located on the outer side of the outer membrane, Hexokinase (HXK), an enzyme that phosphorylates glucose. However, VDAC in complex with CK and ANT does not bind HXK (Vyssokikh & Brdiczka, 2003). ANT is probably functionally coupled to both HXK and CK in these complexes, by supplying ATP used in the phosphorylation reactions, and at the same time disposing of the produced ADP. It is possible that other kinases also take part in similar interactions.

ATP production

The synthesis of ATP is driven by a proton gradient generated by the pumping of protons from the matrix to the IMS. It is the transfer of strong reducing agents (*i.e.* electrons) to a strong oxidant (oxygen) in the respiratory chain, which drives the proton pumping process. The respiratory chain consists of four complexes, in addition to the ATP synthase. Complex I is a NADH dehydrogenase, oxidizing NADH or $FADH_2$ generated in the TCA cycle. This complex is extremely intricate, containing a variable amount of proteins depending on species, with at

least 31 proteins typically being conserved (Cardol *et al.*, 2004). Complex II is succinate dehydrogenase oxidizing succinate to fumarate. This complex also takes part in the TCA cycle. Complex III (cytochrome *c* reductase) transfers electrons from ubiqinol (the carrier of the electrons inside the inner membrane) to cytochrome *c*, the only protein of the respiratory chain not integrally imbedded in the inner membrane. Instead, it is relatively loosely attached to the IMS side of the inner membrane. Intriguingly, in plants, two subunits of complex III, core protein 1 and 2, are identical to subunits of the mitochondrial processing peptidase complex (MPP), which cleaves off presequences form mitochondrial proteins (Glaser & Dessi, 1999). Finally, reduced cytochrome *c* carries the electrons to the last electron acceptor of the respiratory chain, complex IV (cytochrome *c* oxidase). This complex uses the electrons to reduce oxygen to water.

Electron transfer can be uncoupled from ATP production. In plants, alternative oxidases (AOX) catalyse the oxidation/reduction of ubiquinol-oxygen in the absence of proton pumping by complex IV. These enzymes are strongly induced by stresses such as drought and cold, and have been suggested to act as a protection against reactive oxygen species (Costa *et al.*, 2007; Gray *et al.*, 2004; Pastore *et al.*, 2007; Umbach, Fiorani & Siedow, 2005). In addition, rotenone-insensitive NADH dehydrogenases oxidise NADH in the absence of proton pumping. In animals, an uncoupling protein (UCP) has been found that allows protons to flow back into the matrix without the production of ATP. A plant homologue also exist, called plant uncoupling protein (PUMP, Borecky & Vercesi, 2005).

The functions of these different "energy-wasting" mechanisms are not fully known. However, in brown adipose tissue UCP can allow energy to be produced as heat instead of as ATP (Mozo *et al.*, 2005). In plants, PUMP has been suggested to have a role in cold stress defence (Calegario *et al.*, 2003). Uncoupling proteins are found in all eukaryotes, with the exception of yeast. It might be that organisms such as bacteria and yeast, which have high energy demands due to frequent divisions, cannot afford to waste energy in a complete uncoupling process, and therefore no do not exhibit uncoupling proteins. However, yeast does harbour both AOX and alternative dehydrogenases, which may mediate some degree of energy "over-flow" protection.

Genome structure

Being a distant ancestor of the present day *R. prowaskeii*, mitochondria share many similarities with bacteria. However, a big proportion of the original genes have been lost or transferred to the nuclear genome during evolution, leaving mitochondria with genomes 10-1,000 times smaller than that of the early symbiont. Especially animal mitochondria contain a condensed genome, typically ranging between 15-16 kb in size. Plant mitochondrial genomes are larger, containing between 180-2,400 kb, while the genome of the green algae *Chlamydomonas renhardtii* is more like the animal counterpart in this aspect, being 15.8 kb in size (Gray & Boer, 1988). In plants, compared to in animals, large quantities of the genome have no known function. Only 40% of the *A.*

thaliana gene content is accounted for; 10% contain the 57 identified genes while another 30 % contain introns, duplications, integrations of DNA of nuclear and plastid origin and large unidentified open reading frames (Unseld *et al.*, 1997).

Interestingly, some mitochondrial genes appear to have been lost more frequently than others. In most plant species for example, all the genes for the mitochondrial ribosomes are nuclear, while genes encoding subunits of the respiratory chain more often are kept in the mitochondrial genome (Adams & Palmer, 2003). Seemingly, genes coding for proteins with roles in mitochondria specific functions have been retained in the mitochondrial genome more frequently than genes coding for proteins involved in general cell functions. Despite this, the content of some genes in the mitochondrial genome, such as tRNA genes, can vary immensely between species. Yeast mitochondria import only one single tRNA (Martin *et al.*, 1979), whereas kinetoplaztid protozoan mitochondria import all their tRNAs (Hancock & Hajduk, 1990). Plant mitochondria grade somewhere in between on this scale, importing one-half to one-third of the required tRNAs (Kumar *et al.*, 1996). Intriguingly, no tRNAs are imported into mammalian mitochondria or into chloroplasts (Sugiura, Hirose & Sugita, 1998). The big variety in the amount as well as the species of tRNA being imported suggest that the ability to import each tRNA has been acquired at different points of time during evolution.

Mitochondrial protein import

The mitochondrial genomes may encode few genes, but mitochondria contain more than 2000 different proteins (Millar *et al.*, 2005). Most proteins inside the organelle therefore originate from nuclear genes that need to be imported, a process that proceeds via multiple steps (Glaser *et al.*, 1998). In short, the RNA molecule is first translated in the cytosol, producing a precursor protein that most often includes an N-terminal extension, the presequence. This functions as an organellar-sorting and -targeting signal, although targeting information can also be contained in the mature protein (Vergnolle *et al.*, 2005). Secondly, the newly synthesised protein interacts with cytosolic chaperones, such as hsp70, mitochondrial import stimulating factor (MSF) and presequence binding factor (PBF, Hachiya *et al.*, 1994; Komiya *et al.*, 1994; Komiya, Sakaguchi & Mihara, 1996). These help guiding the precursors, as well as keeping them unfolded to facilitate import. Thirdly, the precursor protein is recognized by a receptor located on the outside of the mitochondrial membrane (translocator of the outer membrane, TOM). Electrostatic interactions between the protein and the receptor help guide the newly produced protein. The next step, import through the translocator of the inner membrane (TIM), is dependent on membrane potential, and on ATP. Exceptions to this somewhat simplified scheme exist, *e.g.* regarding the outer membrane proteins, and IMS-localised proteins.

IMS-specific import

Three separate translocation pathways for protein import to IMS have been suggested. Both ATP and the electrochemical gradient drive the translocation of

proteins belonging to class I. In addition, class I proteins contain a bipartite signalling peptide. The first part of this peptide is a mitochondrial targeting signal, which resembles that of the matrix proteins, while the second part contains a hydrophobic sorting domain. It is not known whether the whole protein first is completely translocated into the matrix to subsequently be exported to the IMS compartment, as has been shown *e.g.* for the Rieske iron-sulfur protein in Trypanosoma (Priest & Hajduk, 1996), or if the protein is stopped on the way, with only the targeting part of the presequence actually being transported (Glick *et al.*, 1992). In either case, the signalling part of the presequence is cleaved off in the matrix, exposing the hydrophobic sorting signal at the N-terminal of the unfolded protein. This hydrophobic part of the protein is then inserted into the IMS side of the inner membrane. Finally, an IMS-localised protease cleaves off the peptide chain, allowing the freed protein to adopt proper folding.

Most soluble IMS proteins are imported independently of the inner membrane TIM complex (class II). These proteins are relatively small (typically in the range of 7–15 kDa), and often do not form physical interactions with the TOM complex. Instead they can diffuse freely into the IMS compartment. They also lack a typical mitochondrial presequence, but contain conserved patterns of cysteine (and histidine) residues that enable the binding of cofactors or the formation of disulfide bridges. A disulfide relay system consisting of the Erv1 and Mia 40 proteins can mediate this oxidation (reviewed in Herrmann & Kohl, 2007). Folding of the proteins traps them in the IMS, and it is the energy gain of this process that fuels import. The copper/zinc superoxide dismutase (Sod1) is imported in this way. It remains trapped in the IMS by both cofactor binding and oxidative protein folding (Furukawa, Torres & O'Halloran, 2004; Sturtz *et al.*, 2001).

Class III proteins interact with the TOM complex. After import they fold and stably associate with binding sites inside the IMS on the inner or the outer membrane (Diekert *et al.*, 1999; Steiner *et al.*, 1995). Like the class II proteins, they contain no presequence, although an internal targeting signal was found for heme lyases (Diekert*, et al.*, 1999). In addition, they do not require a membrane potential or ATP for import.

Targeting presequences

Mitochondrial presequences in plants are typically between 10 and 80 amino acids in length, while the animal pre-sequences tend to be shorter (Glaser*, et al.*, 1998). Although attempts have been made to identify a consensus amino acid sequence crucial for targeting, no such sequence has yet been identified. However, some general features have been described. Mitochondrial presequences often contain a high content of hydroxylated basic residues and a low amount of acidic and aromatic amino acids (Glaser*, et al.*, 1998). Interestingly, the N-terminal part of the presequences often consists of a quite regular pattern of alternating basic and hydrophobic residues. This combination gives the peptide the potential to form an amphiphilic α-helix containing one apolar and one positively charged side (von Heijne, 1986). This structure probably guides the precursor protein to the

mitochondrial membrane. It has been suggested that although the α-helix is important for targeting of mitochondrial proteins, it is probably not sufficient (Glaser*, et al.*, 1998). The C-terminal part of the presequence most often contains a processing domain harbouring a motif that directs the cleavage of the presequence from the mature protein.

Mitochondrial processing peptidases (MPPs)

Processing peptidases cleave off the presequence from a mitochondrial protein after import. Although a variety of MPPs acting in the matrix (Gakh, Cavadini & Isaya, 2002), the inner membrane (Esser *et al.*, 2002; Herlan *et al.*, 2003), as well as in the IMS (Esser *et al.*, 2004; Nunnari, Fox & Walter, 1993; Schneider, 1991) have been found in other species, only one plant mitochondrial processing peptidase has been identified to date. As mentioned earlier, the plant MPP is integrated into the cytochrome bc1 complex of the respiratory chain (Braun *et al.*, 1995; Eriksson, Sjoling & Glaser, 1996), while the animal counterpart is a soluble enzyme. Interestingly, cleavage by MPP is very specific, but also general, as it processes a variety of different proteins. In spite of this, no specific sequence identity guiding cleavage has yet been identified, although arginines present at locations –2, -3 or –10 are important (Branda & Isaya, 1995; Glaser*, et al.*, 1998; Zhang *et al.*, 2001). Instead it has been suggested that secondary motifs surrounding the processing site direct cleavage. In *Nicotiana plumbaginifolia* the C-terminal domain of the presequence of the F1-part of the ATP-synthetase was shown to contain a helical segment important for processing (Sjoling, Eriksson & Glaser, 1994).

Interestingly, only organisms harbouring chloroplasts lack a soluble processing peptidase, but have a proteolytically active cytochrome bc1 complex. The reason for this has been suggested to be the unique position of mitochondria in the green cell, which might not require an independent regulation of respiration and protein processing (Brumme *et al.*, 1998; Glaser*, et al.*, 1998). However, the presence of a soluble plant MPP has been detected (Szigyarto *et al.*, 1998; Whelan, O'Mahony & Harmey, 1990), although the identity of the protein is still unknown. In addition, a membrane attached MPP might exist also in animals, as has been suggested before (Gakh, Cavadini & Isaya, 2002). It is therefore possible that the processing mechanisms of animals and plants are not as divergent as first imagined.

Dual targeting

Proteins destined for chloroplasts but translated in the cytosol also harbour targeting sequences referred to as transit peptides. They differ from their mitochondrial counterparts by their lack of positively charged residues, and by the fact that they typically are slightly longer (Zhang & Glaser, 2002). Some proteins, such as tRNA synthetases and RNA polymerases, are directed to both mitochondria and chloroplasts, a phenomenon called dual targeting (Duchene *et al.*, 2005; Hedtke, Borner & Weihe, 2000).

Twin pre-sequences, or so called ambiguous pre-sequences (Peeters & Small, 2001), guide dually targeted proteins to both mitochondria and chloroplasts (Fig. 2). Twin presequences characteristically contain two targeting peptides located in tandem. However, two different proteins are subsequently produced by alternate transcription (Thatcher *et al.*, 2007), translation (Watanabe *et al.*, 2001) or splicing (Zhang *et al.*, 2007). In contrast, ambiguous presequences harbour both targeting signals in one peptide (Hedtke, Borner & Weihe, 2000). These have been found to often exhibit characteristics intermediate in nature between the chloroplast and the mitochondrial targeting peptides. In paper I we discuss the localisation and targeting of NDPK3.

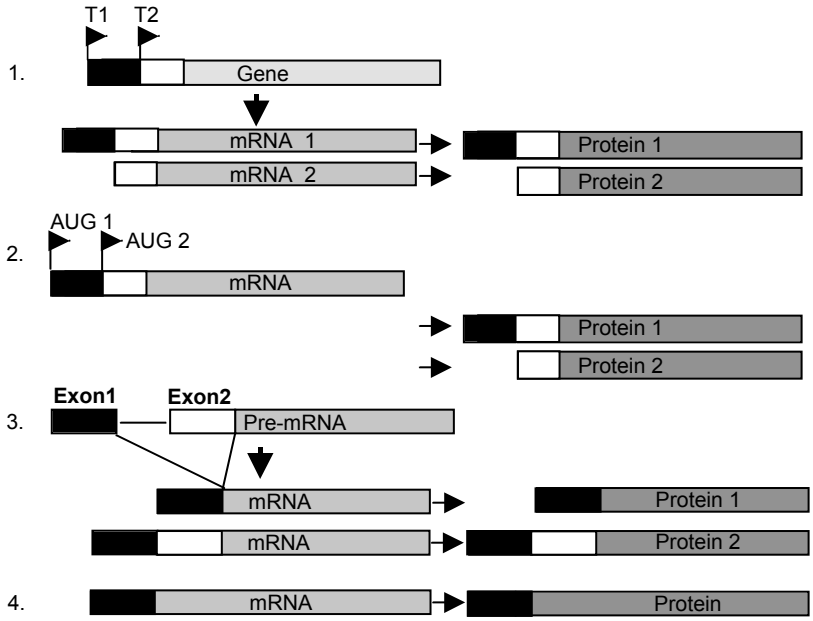

Fig. 2. Illustration of four different ways of achieving dual targeting. 1. Different transcription starts. Two different mRNAs are formed, which will be translated into two different proteins, each having a different targeting signal. 2. Different translation starts. Two start codons (AUG) exist in the mRNA molecule, and consequently two proteins can be produced harbouring different presequences. 3. Alternative splicing. Exons can be excluded from the pre-mRNA message, creating two different mRNA molecules, and two different proteins. 4. Ambiguous targeting signals. One presequence directs import to both mitochondria and chloroplasts. T1, transcription start 1; T2, transcription start 2. Developed from Peters & Small (2001).

Nucleoside diphosphate kinases

NDP kinase activity

The presence of Nucleoside diphosphate kinases (NDPKs) was detected already in the 1950s in yeast and pigeon (Berg & Joklik, 1953; Krebs & Hems, 1953). Most species harbour at least one NDPK gene, although some micro-organisms, such as Mycoplasma species and *Thermotoga maritima* lack this gene altogether (Lacombe *et al.*, 2000). This fact indicates that the NDPK gene is ancient, but not necessary for cell survival. Intriguingly, mutations of the NDPK genes produce quite different phenotypes dependent on species; *Escherichia coli* harbouring a disrupted NDPK gene sustains normal growth, but has a mutator phenotype (Lu *et al.*, 1995), while a point mutation in the *Drosophila melanogaster* NDPK gene causes abnormal wing discs (AWD) and, in combination with the ablation of a phosphodiesterase gene (prune), larval lethality (Sturtevant, 1956). Yeast strains lacking the NDPK gene experience an increased rate of mutations after exposure to DNA damaging agents (Kaetzel *et al.*, 2006). The various effects caused by NDPK ablation early suggested a multi-functionality of the NDPK proteins.

The NDPKs belong to the kinase group of proteins. Kinases are enzymes that transfer phosphate groups from high-energy compounds (most often ATP) to another molecule. This process is called phosphorylation, and can serve to activate or to inactivate a protein. Phosphorylated proteins can also acquire new recognition domains, which might make them able to interact with other proteins. The reverse reaction (the removal of a phosphate group) is catalysed by an enzyme group called phosphatases. The basic metabolic function of the NDPK proteins is to balance the nucleoside pool by the transfer of a phosphate group between nucleoside triphosphates and nucleoside diphosphates. This reaction can be summarized as follows:

$N_1TP + NDPK \Leftrightarrow N_1DP + NDPK\text{-}P$
$N_2DP + NDPK\text{-}P \Leftrightarrow N_2TP + NDPK$ (N = G, A, T, C or U)

In this reaction, a γ-phosphate is transferred to an active residue of NDPK, leaving a nucleoside diphosphate behind. This phosphate is subsequently transferred to a new nucleoside diphosphate, producing a nucleoside triphosphate (Parks & Agarwal, 1973). This catalytic reaction is referred to as a ping-pong mechanism. In this reaction, a histidine residue is binding the phosphate group (Morera *et al.*, 1995). In some cases NDPK phosphorylation has also been observed on serine residues, although this phosphorylation most likely occurs via a mechanism different form that described above. In plants, serine phosphorylation has been reported in *Saccharum offincinarum* L. NDPK (Moisyadi *et al.*, 1994), in *Pisum sativum* (Johansson *et al.*, 2004; Struglics & Håkansson, 1999), and recently in potato (Dorion, Dumas & Rivoal, 2006). Serine phosphorylation has also been observed in *Myxococcus xantus* (Munoz-Dorado *et al.*, 1993).

The significance of NDPK serine phosphorylation has not yet been established, although some reports suggest that it might be part of a signal transduction pathway. In potato, autophosphorylation of S117 has been proposed to act as a

regulatory mechanism of NDPK kinase activity (Dorion, Dumas & Rivoal, 2006). In humans, it was shown that the phosphorylation of a specific serine residue is crucial for NDPK metastasis suppressor activity, and that the actual kinase activity was dispensable in this context (MacDonald *et al.*, 1993). Another signalling function of NDPK has been proposed to be in supplying GTP to GTP-binding proteins. Bonimaar *et al.* found that an NDPK in *Dictyostelium discoideum* is membrane-bound and can activate G-proteins in a cAMP-dependent manner (Bominaar *et al.*, 1993). It has also been reported that autophosphorylated NDPK can phosphorylate GDP bound to G-proteins directly, thereby activating them (Ohtsuki & Yokoyama, 1987).

The presence of a "true" protein kinase activity of the NDPK protein has been debated. Lu *et al.* showed that the *E. coli* NDPK could phosphorylate EnvZ and CheA, which are components of the two-component signal systems in bacteria (Lu *et al.*, 1996). However, this finding was later disputed by Levit *et al.* who demonstrated that this apparent phosphorylation in fact was a product of EnvZ and CheA autophosphorylation (Levit *et al.*, 2002). However, protein kinase activities have been shown for NDPKs of various species, *e.g.* of histone H1 in sugar cane (Moisyadi*, et al.*, 1994) and basic myelin protein in *Bombyx mori* (Uno *et al.*, 2002). Interestingly, also lipid phosphorylation by NDPK has been demonstrated (Muller *et al.*, 2000).

NDPK protein interactions

NDPKs of various species have been found to habitually associate with other proteins. In plants, interactions between the pea NDPK3 and ANT (Knorpp, Johansson & Baird, 2003), as well as between NDPK1 and Catalase 1-3, the latter linking NDPK function to stress detoxification, have been demonstrated (Fukamatsu, Yabe & Hasunuma, 2003). Intriguingly, interactions have also been shown between the chloroplastic NDPK2 and cytosolic proteins such as MAP-kinases (Moon *et al.*, 2003) and phytochrome-A (Choi, Yi & Lee, 1999). In support of the suggested NDPK-phytochrome-A interaction, studies on Arabidopsis NDPK2 knock-out mutants showed defects in cotyledon opening and hook straightening under continuous far-red light (Choi, Yi & Lee, 1999). However, a recent study excludes the presence of this NDPK isoform in the cytosol, suggesting that this interaction might not be valid *in vivo* (Bolter, Sharma & Soll, 2007). It is possible, though, that light can trigger a translocation of NDPK2 to the cytosol, as has been shown for the chloroplastic NDPK in *Neurospora crassa* (Yoshida & Hasunuma, 2006).

Multiple protein partners have also been identified for the mammalian NDPKs. Supporting the view that NDPKs function as suppliers of GTP to GDP proteins, a direct association has been identified between the β-subunit of the G-protein complex and NDPK-H2 (Cuello *et al.*, 2003). In this complex, NDPK-H2 probably transfers a high-energy phosphate from its H118 residue to H266 of the β-subunit of the G-protein, ultimately activating it. Interactions have also been found between NDPK-H1 and hsp70 (Leung & Hightower, 1997), between NDPK-H1 and the PRUNE protein (Reymond *et al.*, 1999), and recently, between

the human Estrogen receptor β and NDPK-H2 (Rayner *et al.*, 2007). Interestingly, NDPKs can also bind kinases. An example of this is the association between NDPK-H1 and the AMP-activated protein kinase in epithelial membranes (Crawford *et al.*, 2006; Muimo, Crawford & Mehta, 2006). In paper IV we investigate the biochemical consequences of the interaction between NDPK3 and the mitochondrial AK.

Human NDPKs

Eight NDPK genes reside in the human genome. These have historically been named nm23-H or nm23, on the basis that a mouse NDPK gene showed strongly reduced expression in metastatic mouse melanoma cells (nm stands for non-metastatic, Steeg*, et al.*, 1988). In this text the human NDPKs will be referred to as NDPK-H1 - NDPK-H8. The human NDPK proteins are involved in proliferation, development and differentiation (Lacombe *et al.*, 2000). However, only NDPK-H1-H4 and H6 exhibit classical NDPK activity. These NDPKs are also expressed ubiquitously, while NDPK-H5, H-7 and H-8 mainly are found in the testis.

NDPK-H1 is a cytosolic isoform, which has been linked to metastasis progression (Hartsough & Steeg, 2000). Especially a low expression of NDPK-H1 could be associated with an increased metastasis potential in several cancer forms (Liu *et al.*, 2005). In breast cancer, the expression of NDPK-H1 was associated with a less malignant tumour type (Horak *et al.*, 2007). However, the reverse relationship has also been demonstrated, primarily in neuroblastoma where NDPK-H1 was found to promote metastasis (Almgren *et al.*, 2004). Several separate molecular activities of this protein could be involved in cancer and/or tumour progression. Firstly, it appears that NDPK-H1 can suppress cell migration and cell motility, which in turn represses the metastatic potential (Horak*, et al.*, 2007; Kantor *et al.*, 1993). Secondly, Fan *et al.* demonstrated that NDPK-H1 is an apoptotic nuclease (Fan*, et al.*, 2003). Thirdly, NDPK-H1 regulates the expression of the PDGF promoter (Ma *et al.*, 2002).

NDPK-H2 is found in the cytoplasm. It also resides in the nucleus, where it can act as a transcription factor with the ability to activate the *c-myc* oncogene (Postel*, et al.*, 1993). In parallel to NDPK-H1, NDPK-H2 has been described to have implications in cell motility as it interacts with ICAP-alpha, a cellular integrin (Fournier *et al.*, 2002). Further, NDPK-H3 harbours an N-terminal signal-peptide, which probably confers the ability of this protein to be secreted (0.648 probability according to a TargetP prediction, http://www.cbs.dtu.dk/services/TargetP/, accessed the 14th of July 2007). Interestingly, the secreted NDPK-H3 has been shown to modulate haematopoietic differentiation (Willems *et al.*, 2002) and not surprisingly, overexpression of NDPK-H3 has been associated with granulocyte differentiation and the induction of apoptosis in myeloid cells (Venturelli *et al.*, 1995). NDPK-H3 overexpression has also been linked to neuroblastoma progression (Amendola *et al.*, 1997).

There are two mitochondrial NDPK isoforms in humans, NDPK-H4 and NDPK-H6. NDPK-H4 is attached to the mitochondrial membranes (Milon *et al.*, 2000; Tsuiki *et al.*, 1999), but in contrast to other mitochondrial NDPKs, no soluble form has been found. It has been suggested that NDPK-H4 could be part of a contact site between the outer and the inner membranes of the mitochondrion (Milon*, et al.*, 2000). As with NDPK-H1, H2 and H3, NDPK-H4 and H6 are also connected to the development of cancer, *e.g.* over-expression of the NDPK-H4 gene was found to be strongly correlated to colorectal tumours (Hayer *et al.*, 2001; Munier *et al.*, 2003). In addition, NDPK-H6, as well as NDPK-H7, have been implicated in colon and gastric cancer (Seifert *et al.*, 2005).

Functions in early spermatogenesis have been proposed for NDPK-H5. Furthermore, the testis-specific NDPK-H8 has an N-terminal that is homologous to thioredoxin. Interestingly, NDPK-related sequences as well as a thioredoxin domain has been found in the sea urcin testis protein IC1, although the significance of this relationship needs to be further elucidated (Padma *et al.*, 2001)

Plant NDPKs

Plants contain three NDPK groups with different subcellular localisations (Escobar Galvis *et al.*, 1999). NDPK1 resides in the cytosol (Tanaka *et al.*, 1998), NDPK2 in the chloroplast stroma (Yang & Lamppa, 1996), and NDPK3 in the lumen of the chloroplast and in the mitochondrial IMS (Spetea *et al.*, 2004; Struglics & Håkansson, 1999; Sweetlove *et al.*, 2001; Yang & Lamppa, 1996). Both a soluble and an IMS-attached form have been identified for the pea mitochondrial NDPK3 protein (Knorpp, Johansson & Baird, 2003). In *A. thaliana* two NDPK3s was found, here referred to as NDPK3a and NDPK3b. These proteins are 93% identical, and the *NDPK3a* and *3b* genes are suggested to be the result of a duplication of an NDPK3 ancestor gene (Paper I). In paper I we characterise and compare the expression of the *NDPK3a* and *NDPK3b* genes.

The biological significance of the plant NDPKs is somewhat elusive, although they appear to often be involved in processes linked to development, stress and/or stress responses. *NDPK1* expression has been shown to be elevated as a reaction to bacterial pathogens in rice (Cho *et al.*, 2004), and the expression of the tomato homologue was found to increase after wounding (Harris, Taylor & Roberts, 1994). The *NDPK1* gene has also been associated with cell growth and division in potato (Dorion, Matton & Rivoal, 2006). NDPK2, on the other hand, has been implicated in the regulation of auxin-mediated respones in growth and development (Choi *et al.*, 2005), and was shown to be able to suppress Bax-initiated cell death in Arabidopsis protoplasts (Baek *et al.*, 2004). Not much is known regarding the biological significance of the mitochondrial NDPK3. However, the interaction with a novel 86-kDa protein during heat shock (Escobar Galvis *et al.*, 2001) suggests a function of this enzyme in stress response. In addition, a decrease in NDPK3 kinase activity in the early stages of programmed cell death was demonstrated in tobacco cells (Valenti *et al.*, 2007). Plant NDPKs have also been implicated in UV-B light signalling (Zimmermann*, et al.*, 1999)

and hormone response (Nato *et al.*, 1997; Novikova *et al.*, 2003; Novikova *et al.*, 1999).

NDPK nuclease activity

The multi-functionality of the NDPKs has at times proved difficult to explain merely on the basis of the well-characterised phosphotransfer mechanism. Many attempts have therefore been undertaken to investigate additional functions of the NDPKs. In the late 1990s Postel *et al*. (Postel, 1999; Postel *et al.*, 2000) showed that the human NDPK-H2 is able to act as a transcription factor for the *c-myc* oncogene by the cleavage of the nuclease hypersensitive region (NHE) in the *c-myc* promoter. By doing so, transcription of the gene was enhanced. Interestingly, NDPK-H1 was shown to bind a similar element in the *PDGF* promoter, although the effect of that binding was inhibitory (Ma*, et al.*, 2002). Since then a range of studies have demonstrated nuclease activity of both human and bacterial NDPKs (Kumar *et al.*, 2005; Levit*, et al.*, 2002; Yoon *et al.*, 2005).

Multiple investigations have suggested that DNA recognition by NDPK occurs via a structure-, rather than sequence-specific mechanism (Kumar*, et al.*, 2005; Levit*, et al.*, 2002; Postel*, et al.*, 1993). Especially the structure of the NHE-promoter motif has been proposed to be important for recognition by NDPKs. It is naturally distorted and forms a chair-like structure, the G-quadruplex (Ambrus *et al.*, 2005; Yang & Hurley, 2006). It is possible that the binding and cleavage of this region by NDPK makes it accessible to conventional transcription factors. The exact mode of action as well as the biological significance of the cleavage still remains elusive, although a fully conserved Lys-12 has been identified as the amino acid responsible for making a covalent bond to the DNA molecule. This residue is located inside the nucleotide-binding cleft of the NDPK structure and has also proved essential for the phosphotransfer activity of NDPK. The finding of a catalytic lysine forming a transiently covalent Schiff-base intermediate (imino-enzyme complex) has suggested the mechanism of DNA breakage by NDPK-H2 to occur via a glycocylase/lyase mechanism (Postel *et al.*, 2002). DNA repair enzymes such as Endonuclease III/Hth and MutM/OOG1 cleave DNA via this route, and consequently DNA cleavage by NDPK-H2 has been proposed to be part of a DNA repair mechanism. However, the function of NDPK as an uracil-DNA glycosylase repair enzyme has been debated (Bennett, Chen & Mosbaugh, 2004; Goswami *et al.*, 2006; Postel & Abramczyk, 2003). In paper II we characterise the nuclease activity of the recombinant mitochondrial pea NDPK protein.

The mitochondrial NDPK and sugar status

The human cytosolic NDPK-H1 is involved in various cellular events concerning sugar and/or energy sensing. One example of this is the promotion of glucose-induced secretion of insulin from islet B-cells by NDPK-H1, which occurs through the activation of G-proteins (Kowluru, Veluthakal & Kaetzel, 2006). In addition, NDPK-H1 can phosphorylate aldolase C that interacts with the GLUT4 glucose transporter and Phospholipase D2 (Wagner & Vu, 2000), two proteins that are involved in insulin regulation of glucose uptake. Seen in this light, it is

probable that NDPK-H1 has functions in the energy sensing mechanism of the cell. Further supporting this notion is the before-mentioned interaction between NDPK-H1 and AMPK. The AMPK protein is a heterotrimer that works as an energy sensor in the cell by, during times of limiting ATP, shutting off processes requiring ATP as well as turning on processes producing ATP. It has been suggested that specific channelling occurs between the NDPK and the AMPK proteins, *i.e.* that NDPK provides ATP specifically to AMPK in a shielded environment. This is logical since AMPK requires ATP in order to phosphorylate down-stream targets at times when ATP produced in the cytosol immediately would be taken up by surrounding processes (Crawford*, et al.*, 2006; Muimo, Crawford & Mehta, 2006).

Sugar metabolism is intimately connected to the mitochondrial compartment through the conversion of pyruvate to ATP. In addition, glycolytic proteins have been identified on the outside of the outer mitochondrial membrane in both plants (Giege *et al.*, 2003) and in mammals (Wilson, 2003). Intriguingly, mitochondrial morphology and size changes and increases, respectively, in response to sugar application in Arabidopsis protoplasts (Hammargren *et al.*, unpublished observations) and in cell cultures (Giege *et al.*, 2005). The ability to convert ATP to GTP, CTP, UTP and TTP gives the mitochondrial NDPKs a potentially central role in the cell's energy metabolism. It is therefore possible that the mitochondrial NDPK also are involved in sugar and/or energy sensing within the mitochondrion.

Sugar signalling in plants

The sugar molecule has a dual function in plants, acting both as a signalling component and as energy. It can therefore be difficult to distinguish between cellular effects created by the sugar molecules as such (sugar signalling) or by a generally elevated energy level (a higher ATP/ADP ratio). Sugar sensing, regulation and signalling have been extensively studied in yeast. Emerging evidence suggests that similar mechanisms are present also in plants (Rolland, Baena-Gonzalez & Sheen, 2006). Various transporters of sucrose and glucose, as well as HXK and sugar receptors, have been proposed to have a role in the sugar sensing machinery. More specifically, sugar sensing in plants have been suggested to pass via an either HXK-dependent or a HXK-independent path (reviewed in Jansson, 2005). According to this model sucrose is either sensed by an unknown disaccharide sensor located on the plasma membrane, or transported via a sucrose transporter into the cell, where Sucrose invertase catalyses the split into glucose and fructose. The glucose molecule is then able to enter the HXK-independent or HXK-dependent pathway of signalling. Glucose can also feed into these pathways via a HXK transporter located on the plasma membrane. This model is further complicated by the fact that HXK-dependent sugar signalling itself can be divided into two separate pathways; one requiring phosphorylation *per se*, and another solely requiring the catalytic activity of the enzyme. Additionally, enzymes that only phosphorylate specific hexoses exist. For example, there are indications that fructokinase could be involved in sugar signalling (Jansson, 2005; Pego & Smeekens, 2000). In paper III we investigate the sugar response of the Arabidopsis NDPK genes, focusing on *NDPK3a*.

Aims of the study

The overall aim of this project was to study the mitochondrial plant NDPKs, focusing on the exploration of potential "new" NDPK3 functions.

Specific aims were to:

Investigate the subcellular localisation of *P. sativum* NDPK3.
Characterise the evolutionary history of plant NDPKs.
Investigate the tissue distribution of the *NDPK3a* transcript in relation to *NDPK1*, *2* and *3b*.
Investigate the potential differences in expression between *NDPK3a* and *NDPK3b* in the flower bud.
Identify proteins interacting with NDPK3 in plant mitochondria and characterise the effect of the interactions.
Identify and characterise IMS-localised nucleases in plants.
Characterise a potential nuclease activity of NDPK3.
Characterise the impact of sugar on the *NDPK3a* gene.

Results and discussion

NDPK evolutionary history and NDPK3 functions related to cell energy status (I, III)

The NDPKs are ubiquitous proteins that have an ancient origin. As well as exhibiting different subcellular localisation patterns, the NDPK genes also are differently expressed. Not surprisingly, transcription of the stromal *NDPK2* is elevated in leaves compared to inflorescence and roots, while the cytosolic *NDPK1* accounts for more than half of the NDPK transcript pool in all investigated tissues. This is in agreement with studies on NDPK1 in potato, in which NDPK1 proved to be the main contributor to NDPK activity in root tips, leaves, tubers and cell cultures (Dorion, Matton & Rivoal, 2006). Interestingly, the second most abundant NDPK transcript is that of *NDPK3a*, while the expression of the highly similar *NDPK3b* gene is hardly detectable. However, a slight elevation in *NDPK3b* transcripts is seen in inflorescence tissue (compared to leaves and roots). Also the *NDPK3a* gene expression is slightly elevated in inflorescences.

In plants, the evolution of different NDPK isoforms predates the emergence of not only monocotyledons and dicotyledons, but also of mosses. Obviously, it is crucial for both plants and animals to harbour different NDPKs in the cytosol, mitochondria and chloroplasts. It is interesting to note that the plant mitochondrial *NDPK3* diverged from the common NDPK ancestor prior to the split between the plastidic *NDPK2* and the cytosolic *NDPK1*. In addition, the *NDPK3* genes are evolutionary more closely related to each other, than are the *NDPK2* genes or the *NDPK1* genes, a fact that could indicate that the *NDPK3* genes have been under a higher evolutionary constraint. This in turn implies that the NDPK3 proteins have a specialised and important function, which may be separate from the cytosolic and stromal isoforms. It is possible that NDPK3, handling and transforming the energy currency ATP inside the main energy producing organelle, has acquired specific crucial functions in energy metabolism, which may be absent from NDPK1 and 2.

Indeed, only the *NDPK3a*, and not the *NDPK1* or *2* genes, is induced by sucrose and glucose in *A. thaliana*. Sugar metabolism is intricately connected with mitochondria through the conversion of sugars to ATP, and through the production of carboskeletons that can be used in anabolic processes. Therefore, it seems logical that the conversion of ATPs into NTPs, and thereby the need for NDPK activity, is accelerated during times of high energy supply, especially in the mitochondrion. An increased energy input also means that the need for NDPs used in anabolic reactions is elevated. Not surprisingly, a high and specific *NDPK3a* expression was seen in rapidly dividing cells in all stages of the developing flower (paper I), a process that can be regarded as highly anabolic. On the other hand, no expression of the second Arabidopsis NDPK3 gene, *NDPK3b*, could be detected in buds up to stage 11, although a distinct expression is present in stage 12 flowers in the tapetum layer and in ovules and petals. It therefore appears that the *NDPK3a* gene is expressed during times of high anabolic activity

(building of new cells and organs), while the expression of the highly similar *NDPK3b* might be more tissue specific, possibly triggered by other mechanisms. In addition, the NDPK3 genes exhibit opposite responses to sucrose application.

Mitochondria have been found to increase in numbers, and possibly also in size, when sucrose is added to cell cultures (Giege*, et al.*, 2005). This raises the question of whether sugar induction of *NDPK3a* also can be seen when the amount of mitochondria is considered, or if the increase in transcripts merely is an adaptation to an elevation in mitochondrial numbers. In the latter case all mitochondrial proteins could be expected to behave similarly. To study this we constructed a custom-made micro-array chip containing cDNAs from about 60 nuclear genes for mitochondrial proteins (J. Hammargren, M. Johansson, P. Bergman and C. Knorpp, unpublished). Preliminary results from this study showed that the induction of NDPK3 after 88 mM sucrose application was 2.6 as compared to the average induction of all genes on the chip, which was 1.6 (J. Hammargren, M. Johansson, P Bergman and C. Knorpp, unpublished). This indicates that the amount of *NDPK3a* transcript increases during sugar application, also when the rise in mitochondrial numbers is taken into account. It is possible that IMS located NDPK fuels GTPases, specifically Mgm1, at times of high energy supply. Mgm1 is needed for inner membrane fusion during mitochondrial division (Meeusen *et al.*, 2006).

Further investigations of the sugar sensitivity of *NDPK3a* showed that equal molar amounts of glucose and sucrose had similar effects, while a sucrose analogue that is not metabolized did not cause an induction. Therefore, we proposed that a possible sugar signalling mechanism involving *NDPK3a* requires sugar metabolism. Further, we demonstrated that sugar induction takes place also in the presence of HXK inhibitor, N-acetyl-glucosamine, suggesting that signalling through HXK does not play a role in the sugar induction of *NDPK3a*. However, sugar signalling leading to *NDPK3a* induction could be HXK-independent, as has been shown in other systems (Hilgarth, Sauer & Tanner, 1991; Martin *et al.*, 1997; Xiao, Sheen & Jang, 2000). Another possibility is that it is the change in energy levels (higher energy) as such, which causes the induction of the *NDPK3a* gene.

In addition, we found sugar related *cis*-elements in the promoter region of *NDPK3a*. These regions have previously been shown to bind a WRKY transcription factor (SUSIBA2) in barley, in a sugar-dependent manner (Sun *et al.*, 2003). In addition, two Arabidopsis WRKY mutants (*AtWrky4* and *AtWrky34*) belonging to the same group of WRKY proteins as SUSIBA2 were shown to have an altered response to sugar on starch metabolism genes (isoamylase 1-3, S. Rosenquist, J. Hammargen, C. Knorpp and C. Jansson, unpublished). Interestingly, this was also true regarding the *NDPK3a* gene, where the *AtWrky4* mutant showed an increased *NDPK3a* expression after sucrose application, while glucose-induced *NDPK3a* expression was decreased in the *AtWrky34* mutant. It therefore seems possible that WRKY transcription factors, at least WRKY4 and WRKY34, are involved in sugar-controlled gene expression also in Arabidopsis.

However, the exact involvement of these genes/gene products in sugar regulation of *NDPK3a* remains to be determined.

Localisation, targeting and interacting proteins (I, IV)

It is well established that the *P. sativum* NDPK3 protein is located in the IMS (Struglics & Håkansson, 1999; Sweetlove*, et al.*, 2001). Specifically, the NDPK3 pea protein has been found both as a soluble and as a membrane-attached form facing the IMS (Knorpp, Johansson & Baird, 2003). Previous studies have demonstrated, by use of salt-washes, that NDPK3 is strongly associated with the inner membrane (Knorpp, Johansson & Baird, 2003). To further elucidate this binding we treated mitoplasts (mitochondria devoid of the outer membrane) with different concentrations of Proteinase K (Fig. 3). By this means we could show that the membrane-bound NDPK is resistant to protease treatment, compared to the cytochrome *c* protein that is a known inner membrane-attached protein facing the IMS. This could mean that the NDPK3 protein is physically protected either by the membrane itself and/or by other interacting proteins. As the same pattern is seen for both pea leaves and potato tubers, this is likely to be a conserved feature of the NDPK3 protein. It is, however, also possible that a portion of the NDPK3 protein is firmly attached to the inner side of the inner membrane, facing the matrix.

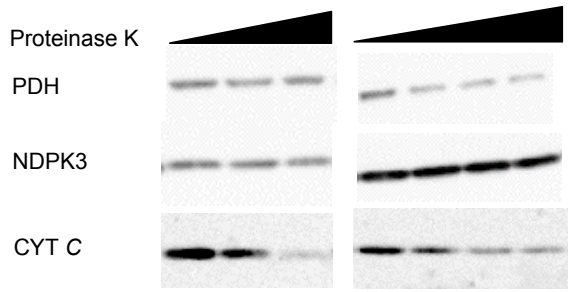

Fig. 3. Western blot showing Proteinase K-treated mitoplasts from potato tuber (left panel) or pea leaf (right panel), probed with antibodies against pyruvate dehydrogenase (PDH, matrix marker), cytochrome *c* (CYT *C*, inner-membrane and IMS marker) and NDPK3. Proteinase K concentrations were between 0-100 µg/ml for potato mitoplasts and between 0-200 µl/ml for pea leaf mitoplasts.

Interacting proteins of the membrane-bound NDPK have been identified, *e.g.* the ANT protein (Knorpp, Johansson & Baird, 2003). Intriguingly, this transporter has previously been shown to associate also with the porin protein as a part of a multiprotein complex (Beutner*, et al.*, 1996). These complexes can, when circumstances allow, form permeability transition pores through which IMS proteins are released during PCD (reviewed in Brdiczka, Zorov & Sheu, 2006). In mammals NDPK has been suggested to be part of such supercomplexes involving ANT and porin (Adams *et al.*, 1989).

In our search for possible interaction partners of the soluble IMS localised pea NDPK3, we used an affinity assay in which covalently bound NDPK was allowed to interact with an IMS-enriched fraction on a column. By this means we identified another protein involved in nucleotide metabolism, AK1 (At5g63400), as an interaction partner of NDPK3. In parallel with the NDPKs, AKs use ATP as a substrate, in the reaction ATP+AMP⇔2ADP. Interestingly, studies have shown that AK is able to complement some of the NDPK function in an *E. coli* null mutant (Lu & Inouye, 1996), although this ability has lately been disputed (Willemoes & Kilstrup, 2005). An association has also been found between the *E. coli* NDPK and AK enzymes inside the phage T4 dNTP synthetase complex (Kim *et al.*, 2005). It might be that similar types of protein assemblies exist in other species, possibly involving nucleotide metabolising proteins. Complexes consisting of nucleotide metabolising enzymes could give these proteins the proximity they need to be able to exert a tight control of the nucleotide balance inside mitochondria.

To further confirm this finding we also performed a pull-down assay, were his-tagged AK1 was bound to Ni-beads, which were incubated with the IMS fraction from pea. After several washes, bound proteins were eluted using imidazole. A western blot from this experiment probed with NDPK3 antibodies is shown in Fig. 4. It demonstrates that although some NDPK3 binding can also be seen in the negative control, a much stronger binding is observed when AK1 is Ni-bound. Interestingly, several NDPK3 bands appear after elution of AK1-bound proteins. These could possibly correspond to different oligomeric forms of the NDPK protein. It seems that despite the presence of denaturating agents in the gel and the loading buffer, the NDPK3 oligomers do not always separate. This is a phenomenon that has been observed at several occasions previously. Gel filtration and crosslinking studies have shown that the NDPK3 protein can be found to form six oligomeric forms (monomer-hexamer). The relative amounts of these forms might vary dependent on condition and function. Johansson *et al.* (Johansson*, et al.*, 2004) demonstrated that the hexameric form is important for the phosphotransfer of pea NDPK3, and Mesnildrey *et al.* have suggested that interactions with other proteins occur at the tetrameric state (Mesnildrey *et al.*, 1998).

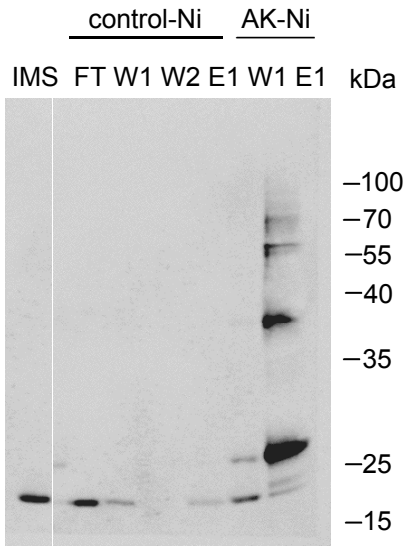

Fig.4. Western blot probed with NDPK3 antibodies showing a pull-down assay. His-tagged AK1 or control protein was attached to a Ni-resin, which was incubated with a pea IMS fraction (100 µg). The resin was then washed and the Ni-attached proteins were eluted with imidazole. 5 µg IMS was loaded in lane 1, all other fractions were loaded as equal volumes of the reactions. AK, Adenylate Kinase 1; IMS, inter membrane space; FT, flow through; W, wash and E, eluate. Myrosinase (EC 3.2.1.147) was used as the control protein. -Ni refers to resin bound protein. PageRuler^{TM} prestained marker (Fermentas) was used.

The *in vivo* significance of the interaction between AK1 and NDPK3 is difficult to predict. *In vitro*, employing a coupled enzyme assay, we found that the presence of NDPK3 in the AK1 assay inhibits AK1 activity. Interestingly, AK1 stimulates the activity of NDPK3. The outcome appears to be an unchanged production of ADP, but with an altered substrate consumption. This means that, at least *in vitro*, the interaction of these enzymes results in a higher production of NTPs in parallel to a lower consumption of AMP. However, other proteins involved in this complex are likely to exist and the possible effects of these interactions remain to be elucidated.

Intriguingly, nucleotide metabolism and NDPK activity has been detected also inside the chloroplast lumen (Spetea*, et al.*, 2004). However, this activity is not due to the chloroplast NDPK2 protein, as this is localised in the chloroplast stroma (Lubeck & Soll, 1995). Indeed, a recent study demonstrated that no NDPK2 protein was present in the luminal space (Sharma, Soll & Bolter, 2007). We investigated this further by use of NDPK-GFP fusion constructs and western blotting, and found that NDPK3 is present in both mitochondria and chloroplasts (*i.e.* it is a dually targeted protein). Therefore, it is possible that the stromal NDPK activity stems from the NDPK3 protein. In support of this view, we found that the chloroplastic form is associated with the thylakoid membranes. These investigations taken together suggest that the NDPK3 protein is attached to the luminal side of the thylakoids, where it could play a role in balancing the

nucloside pool. It is interesting to note that the NDPK2 protein, which is already targeted to the chloroplast, does not translocate into the luminal space (Sharma, Soll & Bolter, 2007). Instead it is the mitochondrial isoform that is present in this compartment. It might be that specific features present in the NDPK3 protein are needed in the lumen, or that the mechanisms of mitochondrial and luminal targeting have evolved together and/or resemble each other.

At least four separate luminal import systems co-exist. These have been denoted Sec-dependent, SRP-dependent, ΔpH/Tat-dependent and spontaneous (reviewed in Robinson, Thompson & Woolhead, 2001). Thylakoid membrane proteins are preferentially imported by the SRP-dependent, or by the spontaneous, pathway. Studies on the LHCP II-protein (apoprotein of the light harvesting complex associated with photosystem II) have shown that SRP-pathway imported proteins normally harbour a stromal transit peptide, and that subsequent targeting into the lumen is dependent on structures within the sequence (Kohorn & Tobin, 1989). On the other hand, proteins that spontaneously insert into the membrane contain two hydrophobic domains at their N-terminal. One of these will form the membrane anchor of the mature protein, while the other is needed during the export from stroma to the lumen, acting as a hydrophobic signalling peptide. The need for such a peptide stems form the fact that proteins imported this way contain a stretch of negatively charged residues at the N-terminal, which otherwise would make membrane translocation unfavourable.

Likewise, protein imported via the Sec-dependent system requires a bipartite signalling peptide. The most N-terminal of these directs import into stroma, while the second part is required for luminal import. Proteins translocated via the Sec pathway is dependent on ATP, but not on a transmembrane potential. In contrast to the single lysine residue that is normally found upstream of the hydorophobic core domain in Sec-translocated proteins, a twin arginine motif is found at this position in proteins translocated via the Tat system (Chaddock *et al.*, 1995). The Tat pathway handles big, fully foled proteins and operates independently of soluble factors.

Interestingly, a processing site for the thylakoid-processing peptidase (TPP) is also found in both the pea NDPK3 protein sequence and in the NDPK3a *A. thaliana* protein sequence (valine at position –3 and alanine at position –1 of the N-terminus of the mature protein). It is therefore possible that this protein is terminally processed in the thylakoids by TPP.

Characterization of nuclease activities present in the IMS

(II and Jenni Hammargren, Carina Knorpp and Per Bergman, unpublished)

In animals, mitochondria also have an important role as an executor in the process of apoptosis, a form of PCD (Green & Reed, 1998; Palmer, Greengrass & Cavalla, 2000). During this event the cell itself governs the death path. This kind of death is "healthier" for the organism than pure destruction of the cells (necrosis) since it recovers energy and does not allow for leakage of molecules, which can cause inflammation in animals. However, it is an energy-dependent process, requiring an active cell. There are two main pathways leading to PCD in mammals, one of which involves the mitochondrion. When death signals converge onto mitochondria, pores eventually form in the outer membrane, allowing the release of molecules such as cytochrome *c*, protease activators and nucleases. At least three different mitochondrially governed PCD processes have been identified. Firstly, mitochondria are able to release cytochrome *c*, which binds the cytoplasmic factor Apaf-1 (Liu *et al.*, 1996). This binding activates a chain reaction involving caspases leading to chromosomal fragmentation and cell death via a caspase-activated DNase (CAD). Secondly, mitochondria can release apoptosis-inducing factor (AIF), which migrates into the nucleus initiating caspase-independent chromosomal fragmentation, a process that normally precedes DNA degradation (Susin *et al.*, 1999). The exact mechanism of AIF action is not known, although it has been proposed that it is part of an endonuclease complex (Wang *et al.*, 2002). Thirdly, a DNA-degrading enzyme called endonuclease G (Endo G) can be released from mitochondria and thereby initiate a caspase-independent chromosomal fragmentation (Li, Luo & Wang, 2001). Endo G cleaves DNA and induces laddering, a well known feature of apoptosis where the nuclear DNA is cleaved into oligonucleosomal fragments of about 50 kb, corresponding to the size of chromatin loops. The next step, internucleosomal cleavage (Oberhammer *et al.*, 1993), has also been ascribed to Endo G activity. Endo G is active at neutral pH, requires Mg and cleaves both RNA and DNA (Widlak & Garrard, 2005).

The involvement of mitochondria in PCD in plants is more elusive. PCD has been found during early embryogenesis (Filonova *et al.*, 2002), during the maturation of anthers (Balk & Leaver, 2001), during cereal endosperm development (Bozhkov & Jansson, 2007), in the formation of root tips, trichomes and vascular tissue, during senescence and during the hypersensitive response reaction (Lorrain *et al.*, 2003). Interestingly, mitochondria do play a pivotal role in PCD during some cases of CMS (Balk & Leaver, 2001; Ku *et al.*, 2003). However, the mitochondrial involvement in PCD in plants remains intangible, although similarities to the animal system exist. For example, plant VDAC has been found to be able to replace animal VDAC in cell death (Godbole *et al.* 2003). Moreover, caspase-like proteins that are up regulated during PCD have been identified in plants (Hoeberichts, ten Have & Woltering, 2003; Hoeberichts & Woltering, 2003). On the other hand, it is not clear whether the release of cytochrome *c* is a key step in PCD in plants, as it is in animals (Balk, Leaver &

McCabe, 1999). Membrane depolarisation, a crucial event in mammal PCD, has been found preceding PCD in plants in some cases (Curtis & Wolpert, 2002), while it has not been detected in other systems (Simeonova *et al.*, 2004). However, based on the mounted evidence it seems possible that mitochondrial components play a role also in plant PCD.

Although no IMS-localised nucleases had been identified in plants previous to our study (paper II), the presence of nuclease activity in the IMS fraction of Arabidopsis mitochondria had been demonstrated. Balk *et al*. (Balk *et al.*, 2003) identified two separate nuclease activities in mitochondria, one that generated chromatin condensation and 30 kb fragmentation within 6 hours, and a second that needed cytosolic extract in addition to mitochondrial extract that induced DNA laddering after incubations of more than 12 hours. The former activity was shown to be Mg-dependent and localised in the IMS fraction.

To follow up on this observation we proceeded to investigate whether IMS nuclease activity is a common feature of all plant mitochondria. To this end, both pea mitochondrial IMS and cauliflower IMS fractions were tested for nuclease activity using an *in vitro* degradation system (Balk*, et al.*, 2003). In this assay the IMS fraction was incubated with either linear or supercoiled Bluescript plasmid in the presence of EDTA or Mg. Using this system, both cauliflower (data not shown) and pea IMS fractions were found to harbour nuclease activity in the presence of Mg (Fig. 5A and 5B). Almost all degradation was abolished when EDTA was added to the reaction, although some nicking activity could still be found using supercoiled plasmid as a substrate (Fig. 5C). Interestingly, at least two separate activities emerged (Fig. 6) when the pH profile of the activity was investigated. One was active at pH between 6.5 and 8.3, but had a dip in activity at pH 7. This activity required Mg. The second activity was found be independent of Mg and was inactive at pH above 7. These profiles could reflect the action of separate proteins. However, it is also possible that they are the mere sum of the activities of two (or more) nucleases present in the IMS of plant mitochondria.

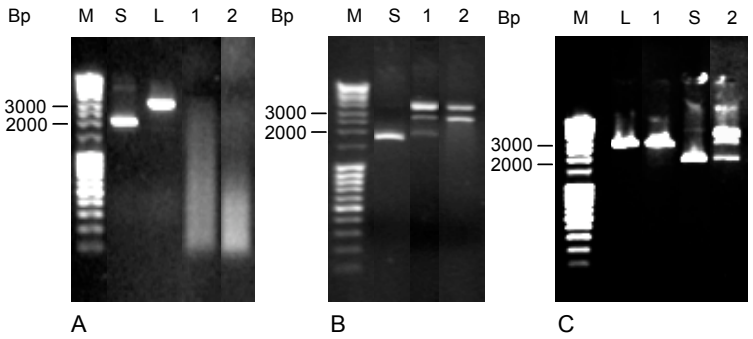

Fig. 5. In vitro plasmid degradation assay. An IMS fraction was prepared from pea leaf mitochondria and 10 µg thereof was incubated with Bluescript plasmid at pH 8.0 at room temperature. A: Incubation of linear (lane 1) or supercoiled plasmid (lane 2) in the presence of 7 mM $MgCl_2$ for 3.5 hours. B. Incubation of supercoiled plasmid in the presence of 7 mM $MgCl_2$ for 15 (lane 1) or 45 minutes (lane 2). C. Incubation of linear (lane 1) or supercoiled plasmid (lane 2) in the presence of 5 mM EDTA for 3.5 hours. M represents DNA ladder (MassRuler™ mix, Fermentas), S and L represent supercoiled and linear plasmid, respectively, incubated for 3.5 hours in the absence of IMS protein.

In an attempt to purify the Mg-dependent nuclease activity a pea IMS fraction was crudely separated on a HiTrap™ Heparin column, using an FPLC system. Proteins were eluted with a linear salt gradient. Two fractions were found to have strong nuclease activity at pH 8.0 in the presence of Mg when tested in the *in vitro* degradation assay (Fig. 7A). The same fractions were separated on SDS-PAGE (Fig 7B). One band appeared only in fraction 27, a fraction that possessed strong nuclease activity. This 39 kDa protein, which eluted at 450 mM NaCl, was cut out and trypsinated followed by QTOF-MS analysis.

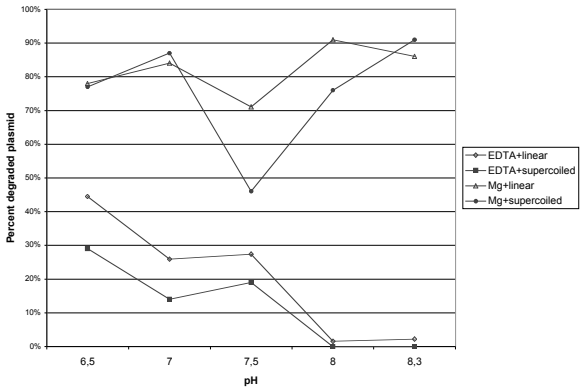

Fig. 6. pH-profile of the nuclease activities located in pea IMS. 250 ng linear or supercoiled Bluescript plasmid was incubated with 10 µg IMS for 3.5 hours at room temperature. 7 mM $MgCl_2$ or 5 mM EDTA was used, respectively. The degradation products were run on agarose gels, photographed and quantified using the Quantity one program (BioRad, v. 4.5.0).

This analysis resulted in 3 pea peptides showing strong similarity to the Arabidopsis Chloroplast stem loop binding protein 41b (CSP41b) amino acid sequence (Fig 8). This protein has not been studied in detail before. However, at the amino acid level CSP41b is 34% identical to and shares 51% similarity with another Arabidopsis Chloroplast stem loop binding protein (CSP41a). CSP41a is a previously studied endoribonuclease that preferably degrades stem-loops in chloroplastic mRNA (Yang & Stern, 1997). Three out of six residues proposed to be important for CSP41a activity, either singly or in combinations (Bollenbach & Stern, 2003), are conserved between the Arabidopsis CSP41a and b (Fig. 8).

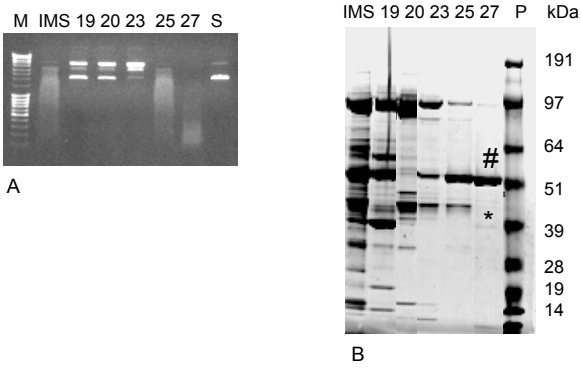

Fig. 7. A 39 kDa protein co-purifies with nuclease activity. Pea IMS was used as starting material for separation on a HiTrap™ Heparin column (GE Healthcare). Crude purification separated the IMS proteins into 3 peaks (excluding the flow-through). A. Fractions 19, 20, 23, 25 and 27 were tested for nuclease activity in the presence of 7 mM $MgCl_2$ at pH 8.0. 4.5 µg protein from each fraction was used except for fraction 20 (7.4 µg), 25 (2.3 µg) and IMS (10 µg). S: no protein added. B. 20 µg of each fraction and the IMS starting material was separated on a SDS-PAGE gel and stained with Commassie-Collodial. M, DNA ladder (MassRuler™ mix, Fermentas). P, protein marker (SeeBlue® Plus2, Invitrogen). Numbers refer to fraction numbers. The asterisk marks the band in fraction 27 that was cut out and sequenced. # marks the major contaminant Serine hydroxylmehyl transferase (SHMT), as determined by QTOF-MS.

Since CSP-proteins to date have been found exclusively in chloroplasts, we were interested in the sub-cellular localisation of CSP41b. Therefore, antibodies were raised against amino acids 353-366 of the CSP41b protein (Fig 8). These amino acids were chosen because they differ between CSP41a and b, in order to ensure specificity of the antibodies. Figure 9 shows a western blot probed with these antibodies, as well as with antisera against two marker proteins, Light harvesting complex IIb (LhcbII) for chloroplasts and Cyt *c* for mitochondria, respectively. Both fractions were found to be highly pure. This enabled us to estimate the distribution of CSP41b protein between the two organellar compartments, concluding that CSP41b is about 7 times as abundant in chloroplast as in mitochondria (data not shown). This might be the reason this protein has not been identified in mitochondria before.

Fig. 8. Protein sequence alignment of *A. thaliana* CSP41a and CSP41b. Text under the two Arabidopsis sequences indicates peptides acquired from MS-QTOF sequencing. Italic letters denote amino acid differing between Arabidopsis CSP41b and the pea sequence. Underlined text shows the peptide used to produce polyclonal antibodies. Bold letters indicate residues important for CSP41a ribonuclease activity (Bollenbach, 2003). Identical amino acids are shown between the CSP41a and b sequences, and similar amino acids are marked by +. The alignment was performed using Blast2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

In order to study in detail the nuclease properties of the CSP41b protein, the gene was cloned and expressed both in *E. coli* and in an *in vitro* transcription-translation system. However, difficulties in the production of the proteins have hindered further investigations of the recombinant protein. In addition, immuoprecipitation studies were not able to deplete IMS fractions of nuclease activity. Therefore, it is possible that CSP41b is not responsible for the observed nuclease activity. However, another scenario could be that CSP41b is just one of several nucleases present in the IMS compartment.

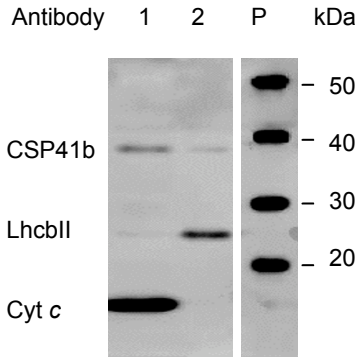

Fig. 9. CSP41b is present in pea leaf mitochondria and chloroplasts. A western blot was probed with antisera directed against a chloroplast marker (LhcbII), a mitochondrial marker (Cyt *c*) and with CSP41b antisera. Lane 1 represents 20 µg pea leaf mitochondria and lane 2 0.5 µg leaf chloroplasts, respectively. P, protein marker (MagicMark™, Invitrogen). LhcbII, Light harvesting complex IIb; Cyt *c*, Cytochrome *c*; CSP41b, Chloroplast stem loop binding protein 41b.

Indeed, the latter scenario seems to be correct. We found that the IMS-localised pea NDPK3 protein exhibits a non-specific endonuclease activity that is active towards both DNA and RNA molecules. Interestingly, nucleic acid molecules harbouring specific secondary structures appear to be favoured NDPK3 targets as demonstrated by the inability of NDPK3 to cleave RNA and DNA molecules, which are predicted not to have a stable secondary structure. Supercoiled plasmid DNA is cleaved into the open circular and linear forms. Also tRNA molecules as well as the 3'UTR of the *ATP9* gene were cleaved by NDPK3. The structure and/or sequence specificity of the NDPK nuclease activities have been a matter of debate. However, as mentioned before, the secondary structure of the nucleic acid molecules are suggested to be the primary determinant for NDPK cleavage specificity.

In addition, NDPK3 cleavage activity was independent of the ability to form a hexameric structure or of a functional phophorylation at histidine H117, as shown by mutant studies. It was also inhibited by ATP, in parallel with what has been demonstrated for human NDPK-H2, *E. coli* NDPK and for *Mycobacterium tuberculosis* NDPK (Kumar*, et al.*, 2005; Levit*, et al.*, 2002; Postel, 1999). Interestingly, tRNA import experiments performed in the absence of ATP yielded fragments identical in size to those seen after NDPK incubation. The procedure of the import experiment is explained in Figure 10A, and the import experiment is presented in fig 10B. It is possible that NDPK3 could act as some kind of tRNA availability control, which potentially would stall the production of new transcripts during times of low energy (low ATP). However, we failed to detect NDPK3 in the soluble matrix fraction (data not shown), indicating that cleavage occurs prior to, or during the process of translocation. It is possible that NDPK interacts with a, to date unknown, import protein located in the mitochondrial

inner membrane. The potential involvement of NDPK3 in the PCD process, however, calls for further investigations.

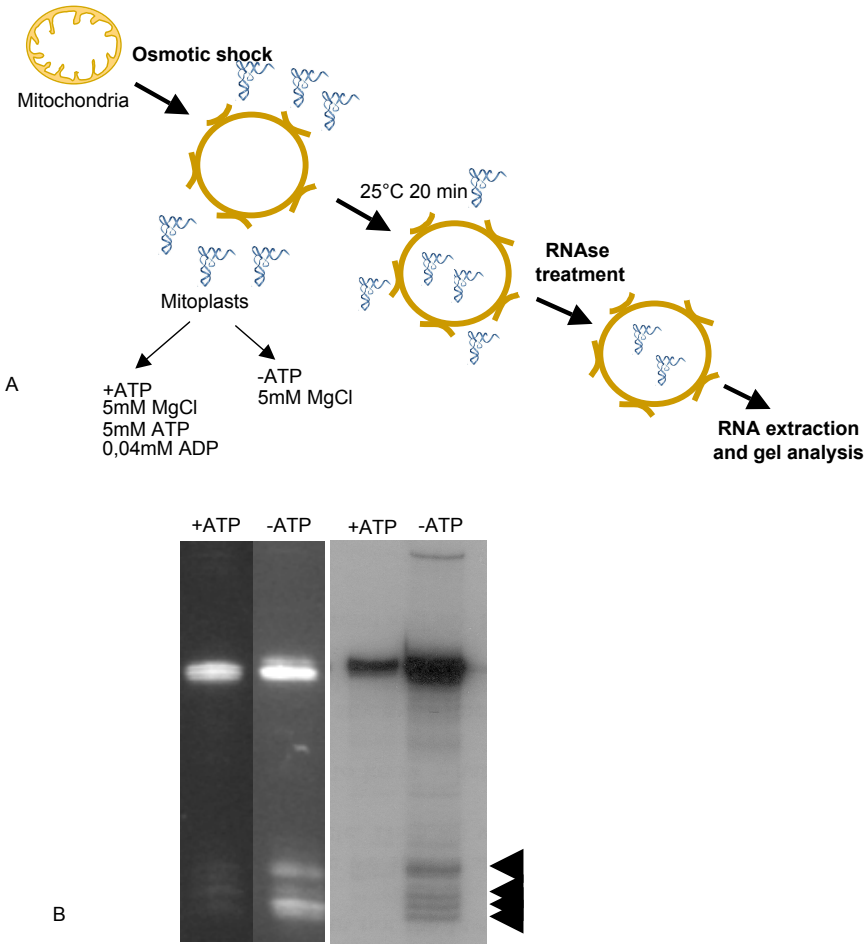

Fig. 10. A shows the course of a tRNA import experiment. Blue "ribbons" illustrate the tRNA molecules. The left panel in B shows the separation of $tRNA^{Ala}$ degradation products on an agarose gel after incubation of 600 ng NDPK3 with 250 ng tRNA at 30°C for 2.5 hours in the presence and in the absence of 2mM ATP. The right panel shows an autoradiogram of an import experiment conducted in the presence and absence of 5mM ATP. Arrows mark degradation products.

Future perspectives

Paper I

We demonstrated that NDPK3 is dually targeted, *i.e.* localised to both the IMS and the thylakoid compartment of the chloroplasts. However, the function of this protein in the thylakoids is still unknown. Proteins involved in the phosphorylation of thylakoid targets in response to light have been identified previously, and are important for the photosynthesis process (Lundin *et al.*, 2007; Zer & Ohad, 2003). As protein kinase activity has been demonstrated for some NDPKs, NDPK3 could be involved in the phosphylation of thylakoid proteins. Interestingly, degradation of photo damaged photosystem II proteins during photoinhibition has been shown to be dependent on GTP, which could be supplied by the thylakoid-localised NDPK3 (Spetea *et al.*, 1999). However, to properly investigate the possible involvement of NDPK3 in these processes, it is crucial to first in detail determine the sub-chloroplastic localisation of NDPK3. To this end, fractionations of thylakoids and lumen should be carried out followed by western blotting. In order to identify possible targets of the chloroplast localised NDPK3, phosphorylation studies using thylakoid membranes and overexpressed NDPK3 can then be performed. In addition, to further study the function of the chloroplast-localised NDPK3, possible interaction partners should be searched for, *e.g.* by use of blue-native gels. In order to evaluate the effect *in vivo*, investigations involving Arabidopsis plants overexpressing and underexpressing the NDPK3 protein can be undertaken with respect to chloroplast specific functions, *i.e.* photosynthesis.

Furthermore, the processing peptidase/s acting on the mitochondrial NDPK3 after import is still unknown. However, the NDPK3 protein sequence harbours sequences that could be used as motifs for the TPP. It is therefore possible that the mitochondrial processing of this protein occurs via a mechanism involving a homologue to the TPP as has been suggested before (Nunnari, Fox & Walter, 1993), although this protein still remains to be identified. *In vitro* processing experiments using mutated NDPK proteins could clarify this question. Candidate amino acids to start investigating would be the Sec system-specific residues present in NDPK3; valine at position –3 and alanine at position –1 with respect to the processing site.

Paper II

In paper II we showed that the recombinant pea NDPK3 enzyme is an IMS-localised nuclease. Interestingly, mammalian nucleases located in this compartment often take part in the PCD process. Therefore, it would be of interest to further investigate the possible involvement of the pea NDPK3 in nucleic acid degradation during plant PCD. An important feature of PCD nucleases is their translocation from the IMS to the nucleus during cell death. To elucidate whether the NDPK3 protein takes part in such a translocation process, protoplasts and/or whole plants transformed with NDPK3-GFP could be heat shocked to induce PCD

(Balk, Leaver & McCabe, 1999), and the location of the NDPK3 fusion protein further studied during the death process. Alternatively, NDPK-antibodies labelled with a fluorophore could be incubated with fixed cells undergoing PCD to study the course of NDPK3 movement in the cell. Another feature of PCD is chromatin condensation and nuclear DNA degradation. In order to investigate these processes in regard to the NDPK3 protein, nuclear preparations may be incubated with recombinant NDPK3 and subsequently separated on agarose gels. Preparations of pure nuclei can also be used to confirm whether NDPK3 is present in the nucleus after heat-induced cell death. Further, cross-linking of proteins associated with DNA would reveal if NDPK3 indeed is associated with DNA *in vivo*.

Further, experiments using mutated nucleic acid substrates can give answers as to the specificity of NDPK3 nucleic acid binding and cleavage. In addition, mutagenised recombinant NDPK3 could be employed in order to identify the amino acids involved in cleavage and binding. The conserved Lys-12, which has been shown to form the covalent bond to DNA in human systems, is a candidate amino acid to be mutated. The finding of NDPK3 amino acids, which separate the cleavage activity of NDPK3 from the phosphorylation activity (*i.e.* residues of importance for the DNA cleavage activity but not for phosphorylation), is also of great interest. In addition, incubation of recombinant NDPK3 mutant proteins with various DNA substrates would reveal the nature of the ATP inhibition, *e.g.* the H117A mutant could be studied in relation to the importance of active site histidine phosphorylation for the inhibition effect.

As described earlier, some NDPKs have been shown to act as transcription factors. In this light, the possible interaction of NDPK3 with promoters may be studied by band-shift assays, and the effect of the binding could be studied using reporter systems (*e.g.* luciferase assays).

Paper III

We demonstrated an effect of sugar on the expression of the *NDPK3a* gene. However, it is still not clarified whether the observed change is due to a general increase in energy (*i.e.* more ATP present) or to some sort of specific sugar signalling. To further elucidate this, extended expression studies using multiple sugars, *e.g.* fructose, should be performed. The effect of sugar application and sugar starvation on the *NDPK3* gene *in vivo* could also be studied with a focus on energy requiring processes, *e.g.* budding and sprouting, employing homozygous knock-out Arabidopsis plants. In addition, the expression of the *NDPK3a* gene was seen to increase, while the total NDPK3a protein amount did not, indicating that only newly produced protein is active. Investigating the fate of the newly produced NDPK, as well as the rate of NDPK protein synthesis after sugar application, would therefore be of importance. This could be done by conducting pulse-chase experiments.

The evolutionary origin of different NDPK isoforms is ancient. However, it is highly likely that functions and regulation of the same isoform have adapted to the

specific requirements of different species. In order to determine the evolutionary descent of the *NDPK3a* sugar induction, the effect of sugars on the mitochondrial NDPK gene in other species, *e.g.* mosses, could also be investigated. Further studies should also be conducted regarding the transcription factors regulating the *NDPK3a* gene in a sugar-dependent manner. To this end, sugar regulation of *NDPK3a* by WRKY proteins could be investigated by means of reporter systems. Band shift assays using recombinant WRKY4 and 34 proteins incubated with the *NDPK3a* promoter could confirm binding.

Paper IV

NDPK proteins of various isoforms and species have been shown to work in complex with other proteins. NDPKs can probably have multiple functions in such complexes. One task would be to regulate the activity of the interaction partners, while another could be to supply NTPs for interacting proteins. We found a connection between the mitochondrial AK1 and NDPK3. When the activities of these proteins were measured in the same assay, NDPK3 was found to inhibit the effect of AK1, while AK1 was observed to stimulate the activity of NDPK3. However, the association between these proteins should be further confirmed. This could be done by studies using the previously described pull-down assay and/or by the incubation of a pure IMS fraction on a column containing covalently bound AK1. Interaction studies *in vivo* could also be conducted using *e.g.* bimolecular fluorescence complementation (BiFC, Walter *et al.*, 2004), although there is a possibility that this study may fail due to steric hindrance (see paper IV). In addition, raising antibodies against the mitochondrial AK1 isoform would be crucial since this would facilitate the study of the sub-mitochondrial localisation of AK1 in greater detail. This would also provide the opportunity to perform reciprocal immunoprecipitaion studies on AK and NDPK3. Further, the identities of possible additional members of the complex could be identified by one of these methods.

Concluding remarks

This thesis illustrates the complexity and multi-functionality of the plant NDPKs. We suggest that despite the similarities between the NDPK1, 2 and 3 proteins, they have specialized functions in their respective compartment, which developed prior to the divergence between mosses and higher plants. We also propose that energy availability (possibly in connection to sugar signalling) as well as anabolic activity, are important factors for the expression of the mitochondrial NDPK3 gene. The mature NDPK3 protein may exert many of its effects in cooperation with other proteins, probably as part of multi-protein complexes. The interesting finding of NDPK3 in the chloroplast thylakoids indicate possible links between, or shared functions of, IMS NDPK3 and NDPK3 in the lumen and/or thylakoids. Considering the amazing divergence in NDPK properties, a role for NDPK3 as a key-regulator, possibly transferring signals between the nucleus and the mitochondrion, could be envisioned.

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