Phosphorylation of chloroplast preproteins and the characterisation of nucleoside diphosphate kinase 2

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Dedicated to my spiritual guru.

Learning

is finding out

what you already know.

Doing is demonstrating that

you know it.

Teaching is reminding others

that they know just as well as you.

We are all learners,

doers, teachers.

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Abbreviations

aa	Amino acid
BSA	Bovine serum albumin
CAO	Chlorophyll A Oxygenase
C-terminus	Carboxyl terminus
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FNR	ferredoxin-NAD(P)+ oxidoreductase
GFP	Green flourecent protein
HEPES	N-2-hydroxyethypiperasin-N'-2-ethasulfonate
HCF	High Chlorophyll Fluorescence
Hsp	Heat shock protein
IPTG	Isopropylthiogalactoside
kD	kilodalton
LHCP	Light Harvesting Complex Protein II
Met	Methionine
MS	Murashige and Skoog
MOPS	Morpholinopropansulfonate
mSSU	Mature protein of the small subunit of Rubisco
N-terminus	Amino terminus
NBT	4-nitrotetrazoliumchlorid-blue-hydrate
NDPK2	Nucleoside diphosphate kinase 2
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethane sulfonyl fluoride
pOE23	23-kDa oxygen evolving complex subunit
pOE33	33-kDa oxygen evolving complex subunit
pSSU	Precursor protein of the small subunit of Rubisco
Rubisco	Ribulose-1,5-bisphosphate carboxlase/oxygenase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SiP	Ferredoxin :sulfite reductase
TCA	Trichloroacetic acid
Tic	Translocon at the inner envelope of chloroplast
Toc	Translocon at the outer envelope of chloroplast
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-glycin
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

1. Abstract

The nuclear-encoded preproteins destined for chloroplasts are phosphorylated at a serine or threonine residue in their transit peptides by a cytosolic protein kinase. A phosphorylation motif was proposed for these sites of phosphorylation. In the present study, the sites of phosporylation were investigated for the proteins APC1, HCF136, CAO of *Arabidopsis thaliana* and pea LHCP. In case of APC1 and LHCP, it was found that more than one site of phosphorylation exist in their transit peptides. For HCF136, the actual site of phosphorylation differred from the site determined by the proposed phosphorylation motif, whereas the transit peptide of CAO was not found to be phosphorylated at all. Further, it was tried to find the significance of this phosphorylation on the import process, *in vivo*. The attempt with microinjection technique remained unsuccessful due to methodological drawbacks, whereas with *in vivo* labelling technique, a difference between the imported proteins was observed for the wild type and the mutated form of OE23. The second part of this work involved the study of the isoform of the ubiquitous housekeeping enzyme, the Nucleoside diphosphate kinase (NDPK2). The NDPK2 from pea had been earlier reported to have two forms in the chloroplast. The detailed biochemical characterisation of these forms was taken up here. No difference between their import behaviours and localisations within chloroplasts could be determined. They were both found to be in the stroma, associated with thylakoids and nucleoids. In recent years, a number of groups have reported the presence of the NDPK2 of *Arabidopsis thaliana* to be in the nucleus and cytosol which contradicts with earlier studies conducted with pea, spinach and *Brassica campestris* where this isoform was found to be in chloroplasts. The present work shows that NDPK2 from *Arabidopsis thaliana* is also localised within the chloroplasts.

2. Zusammenfassung

Kernkodierte chloroplastidäre Vorstufenproteine werden von einer zytosolischen Proteinkinase an einem Serin- oder Threoninrest innerhalb des Transitpeptides phosphoryliert. Ein (Erkennungs-) Motiv für diese Phosphorylierungsstelle wurde bereits postuliert. In der vorliegenden Arbeit wurden die Phosphorylierungsstellen der Proteine APC1, HCF136 und CAO aus *Arabidopsis thaliana* und LHCP aus *Pisum sativum* untersucht. Für APC1 und LHCP konnte gezeigt werden, dass mehr als eine Phosphorylierungstelle im Transitpeptid vorliegt. Die Phosphorylierung von HCF136 fand an einer anderen Stelle als anhand des bekannten Motivs vorhergesagt wurde statt. Das Transitpeptid von CAO wurde hingegen nicht phosphoryliert. Des weiteren wurde die Signifikanz der Phosphorylierung für den Importprozess *in vivo* untersucht. Die Methode der Mikroinjektion erwies sich auf Grund methodischer Schwierigkeiten als ungeeignet, wohingegen die *in vivo* „Labeling" – Technik einen deutlichen Unterschied zwischen dem importierten OE23-Wildtyp-Protein und der mutierten Form zeigte. Der zweite Teil dieser Arbeit beschäftigte sich mit der Isoform 2 des ubiquitär vorkommenden Enzyms Nucleotid Diphosphat Kinase (NDPK2). Für NDPK2 aus Erbse wurden bereits zwei Formen in Chloroplasten identifiziert. Diese sollten im Detail biochemisch charakterisiert werden. Es zeigten sich keinerlei Unterschiede hinsichtlich des Importverhaltens und der Lokalisation innerhalb der Chloroplasten. Beide Formen befinden sich im Stroma, wo sie mit Thylakoiden und Nukleoiden assoziiert sind. In den letzten Jahren haben einige Arbeitsgruppen die Anwesenheit von NDPK2 in *Arabidopsis thaliana* im Kern sowie im Zytosol beschrieben. Dies widerspricht früheren Veröffentlichungen über Erbse, Spinat und *Brassica campestris*, die diese Isoform der NDPK in Chloroplasten lokalisierten. In der vorliegenden Arbeit konnte gezeigt werden, dass NDPK2 aus *Arabidopsis thaliana* im Chloroplasten lokalisiert ist.

3. Introduction

Oxygenic photosynthesis is the process by which energy from sunlight is transformed into biochemical energy in the form of ATP and NADP(H), and CO_2 along with H_2O are converted into carbohydrates and O_2. The apparatus responsible for this process is localised in the thylakoid membrane system in plants, algae and oxygenic bacteria. In cyanobacteria it is built up of long lamellae that enclose an aqueous compartment called the lumen. In eukaryotic oxyphototrophs, it is localised in organelles known as chloroplasts. This capability of chloroplasts in carrying out oxygenic photosynthesis, which is the major source of atmospheric oxygen, indicates towards its significance to the existence of life on earth (Fig. 1).

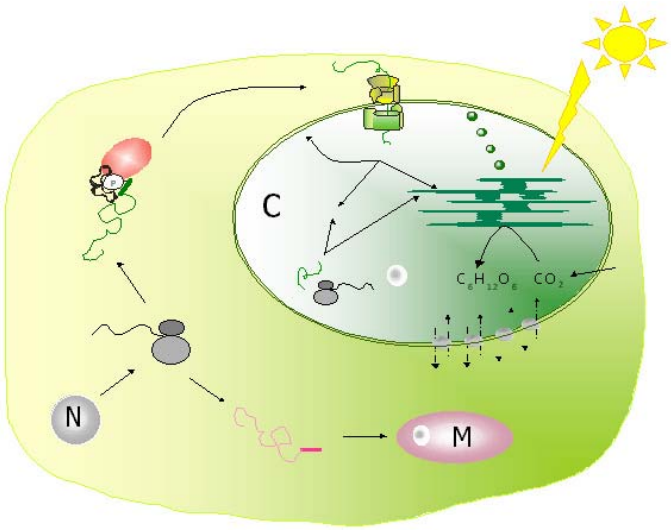


Figure 1. A photosynthesizing plant cell depicting the nuclear-encoded proteins being transported into chloroplast and mitochondria. C-Chloroplast, N-Nucleus, M-Mitochondria, P-Phosphorylated preprotein (Vothknecht and Soll, 2002).

Eukaryotic cells are highly compartmentalised. They contain at least ten different compartments made up of several membrane-bound organelles. The chloroplast is one such organelle which belongs to the family of organelles called plastids. Chloroplasts are believed to have evolved from ancient cyanobacteria (autotrophic prokaryotes) in an endosymbiotic process (Margulis, 1970). According to the now widely accepted endosymbiont theory, an ancient amoeboid eukaryote engulfed a cyanobacterium, which lost its independence by massive gene transfer to the nucleus of the eukaryote, and subsequently developed into chloroplasts (Lopez-Garcia and Moreira, 1999; Martin *et al*., 2002). In effect, they exist now as semi-autonomous organelles, i.e. they contain their own DNA to code for some of their proteins and the ribosomes necessary for the translation of appropriate mRNAs, but most of their proteins are encoded by the nuclear genome. For example, in *Arabidopsis thaliana,* the chloroplast proteome has been estimated to have around 2500 proteins, but only about 87

are estimated to be coded by the chloroplast genome (Abdallah *et al*., 2000). The rest are encoded by the nuclear genome. The nuclear-coded proteins are translated in the cytosol and then transported into chloroplasts. Therefore, for the proper understanding of the functioning of a compartmental structure, as found in eukaryotic cells, it is crucial to study the transport machineries that are in effect within the cell.

The sorting of proteins in the cytosol is an accurate process and is mediated by the necessary sorting machinery present within the cell which directs each protein to its proper destination. Proteins destined for chloroplasts are imported post-translationally. Their folding to form biologically active proteins is prevented in the cytosol by molecular chaperones (Jackson-Constan *et al*., 2001). They acquire their mature functional forms only when they reach their respective locations within the chloroplast. Most of the chloroplast-bound proteins contain one or more targeting domains that act as an address label for its final destination. These targeting domains are mostly short peptides or amino-acid motifs that are located mainly at the amino-(N-) terminal end of the protein and are referred to as the transit peptides (Glaser and Soll, 2004). Specific cellular machineries interact with this information to target the protein to the chloroplast surface and its subsequent transport across the two envelope membranes of the chloroplast. Most of the proteins are translated with a cleavable transit peptide and enter the chloroplast by a general import pathway which is mediated by two multisubunit protein complexes of the chloroplast envelope designated as the Toc (Translocon at the outer envelope of chloroplasts) and the Tic (Translocon at the inner envelope of chloroplasts) complexes, respectively (Jarvis and Soll, 2002). However, a subset of the chloroplast targeted proteins do not contain transit peptides and are translated in the cytosol in their mature forms (Salomon *et al*., 1990; Kessler et al., 1994). This group includes nearly all integral membrane proteins of the outer envelope (OEPs, outer envelope proteins) and at least two inner envelope proteins, namely, ceQORH (Miras *et al*., 2002) and Tic32 (Nada and Soll, 2004). After reaching the stroma, the transit peptide is cleaved off by stromal processing peptidase (Richter and Lamppa, 1998). Thylakoid-destined proteins contain a bipartite transit peptide. Its stromal-directing peptide is removed in the stroma whereas the thylakoid-directing peptide is cut by the thylakoid processing peptidase in the thylakoid lumen (Chaal *et al*., 1998). A protein containing a cleavable transit peptide is referred to as a preprotein. The preproteins are thus larger in size than their mature forms and hence can be differentiated by various techniques.

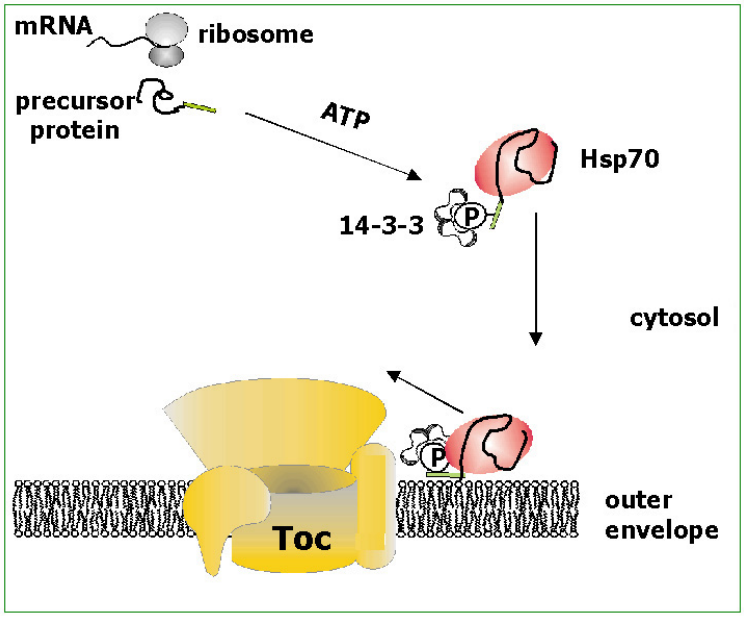


Figure 2. Transfer of preproteins from the cytosol to the chloroplast membrane by the guidance complex comprising of chaperone Hsp70 and a 14-3-3 P-binding protein.

While still in the cytosol, the preproteins require a number of soluble cytosolic factors to translocate them to the chloroplast surface. One such factor is a kinase which was reported to phosphorylate some prominent chloroplast destined preproteins in an ATP-dependent manner. The enzyme was found to be plant specific and the phosphorylation of the preproteins was shown to occur at either a serine or a threonine residue in the transit peptide of the preprotein (Waegemann and Soll, 1996, Su *et al*., 2001). This phosphorylation results in the binding of the preprotein to a cytosolic guidance complex which in turn has a stimulatory effect on the import rate of the preprotein. The guidance complex consists of a Hsp70 chaperone, a 14-3-3 dimer and maybe a few other proteins (May and Soll, 2000) (Fig. 2). 14-3-3 proteins recognise and bind to phosphorylated proteins, whereas Hsp70 association seems to be required to keep the preproteins in an import competent unfolded state (Soll and Schleiff, 2004). The precursor-guidance complex docks at the primary receptor and is then further translocated by the Toc machinery. Precursor proteins present in the guidance complex are imported into chloroplasts at much higher rates than monomeric preproteins. Therefore, phosphorylation and guidance complex formation can represent an important regulatory circuit in an early step of protein import. The transit peptides of preproteins have been suggested to contain a loosely conserved phosphorylation motif $(P/G)X_n(R/K)X_n(S/T)X_n(S^*/T^*)$, where 'n' denotes zero to three amino acids spacer and S*/T* represents the phosphate acceptor. This motif has been established by finding the sites of phosphorylation for tobacco pSSU, pea pOE23 and pea pOE33 proteins by site-directed mutagenesis (Waegemann and Soll, 1996).

Our present knowledge regarding the above mentioned information is based on *in vitro* experiments. The general experimental set up for such a study comprises of the translation of mRNA from the cDNA of one organism either in a wheat germ or reticulocyte lysate system (representing the second organism), and further the import of these proteins into chloroplasts isolated from a third organism. This heterologous experimental set-up might yield varied results as compared to *in vivo* experiments (Rudhe *et al*., 2002). In order to address this problem, *Arabidopsis thaliana* was chosen in the present study as a system to elucidate the significance of phosphorylation of preproteins in the cytosol during the import process. For this purpose, three proteins from *Arabidopsis thaliana* were chosen to study their sites of phosphorylation. These proteins were APC1 (*atpC1*), HCF136 (*hcf136*) and CAO (*atcao*). APC1 is a gamma subunit of ATP-synthase, HCF136 is related to the assembly of Photosystem II in chloroplasts and CAO is a chlorophyll a oxygenase. These proteins were chosen for the experiments as they show clear phenotypes in their knock out mutant plants which are available in-house (HCF136- Meurer *et al*., 1998; APC1- Bosco *et al*., 2004; CAO- Meurer *et al*., unpublished data). The first part of this study consisted of the localisation of the sites of phosphorylation for these three proteins, followed by an attempt to establish a homologous system to study the effect of phosphorylation upon the import process.

Another essential requirement for the viability of a cell, in addition to the transport of nuclear-encoded proteins to their respective compartments, is the constant supply of nucleotides. Nucleotides are needed for various ongoing processes like phosphorylation, translocation, transcription etc.. One of the well studied enzymes which is known to generate nucleoside triphosphates (NTPs) in the cell is the Nucleoside diphosphate kinase (NDPK). It is a ubiquitous enzyme found in organisms from bacteria to mammals and plant species. The first NDPKs were discovered over 50 years ago in pigeon breast muscle (Krebs and Hems, 1953) and yeast (Berg and Joklik, 1953). Since then a number of genes encoding NDPKs have been identified and characterised in a variety of organisms, including humans (Postel *et al*., 1993), rats (Kimura *et al*., 1990), *Drosophila*, yeast (Fukuchi *et al*., 1993), *Dictyostelium* (Troll *et al*., 1993), and bacteria (Munoz-Dorado *et al*., 1990). The NDPKs comprise a highly conserved family in eukaryotes (Lacombe *et al*., 2000). They show remarkable sequence conservation and have identical active residues. Their structures resolved by X-ray crystallography also show high similarity.

In humans, eight genes coding for different NDPK isoforms have been reported which have been named as *nm23-H1* to *nm23-H8*. They derive their names from the first *nm23* cDNA which was obtained by screening human fibroblast cDNA library with murine *nm23* cDNA isolated on the basis of its reduced expression in highly metastatic murine melanoma

cell lines (Steeg *et al*., 1988). The human NDPK proteins on the other hand are designated in the literature as NDPK A to NDPK H. In plants, their nomenclature is much more varied. In *Arabidopsis thaliana*, they are referred to as NDPK1 to NDPK4. The recently characterised NDPKs of *Brassica campestris* are named as BcNDK1 to BcNDK3 (Dong *et al*., 2004), whereas the NDPKs from pea, rice and spinach have been referred to as NDPK I to NDPKIII. Thus there appears to be a high level of inconsistency in the nomenclature of NDPKs in the literature. For the purpose of convenience, we propose the use of the terminology in the series of 1, 2, 3 and so on, with the prefix containing the abridged form of the generic and the specific name of the concerned species. For example, atNDPK1, atNDPK2, atNDPK3, atNDPK4 for the NDPKs of *Arabidopsis thaliana* and psNDPK1, psNDPK2, psNDPK3 for those of *Pisum sativum*. This nomenclature will be followed in the present study.

As mentioned above, eight isoforms of NDP kinase have been found in humans. In contrast, only three isoforms are encoded by the *Drosophila* genome (Lacombe *et al*., 2000). In plants, three to four isoforms have been reported. These isoforms are targeted to different subcellular locations. In the animal kingdom, they have been found in the cytoplasm, mitochondria and nucleus. In plants, in addition to these locations, NDPKs have also been reported to be present in the chloroplast. In general, NDPK1, which lacks an identifiable targeting sequence, is reported to be in the cytosol, whereas NDPK2 and NDPK3, having N-terminal extensions are reported to be in chloroplasts/cytosol and mitochondria, respectively. NDPK1 has been characterised and cloned from spinach (Nomura *et al*., 1991; Nomura *et al*., 1992), pea (Finan *et al*., 1994), rice (Yano *et al*., 1995), *Brassica campestris* (Dong Ho Shin *et al*., 2004) and potato (Dorion *et al*., 2006). NDPK2 has been isolated from chloroplasts of pea (Luebeck and Soll, 1995) and spinach (Yang and Lamppa, 1996). Several studies indicate the presence of the NDPK3 isoform in the intermembrane space of mitochondria (Bessman and Carpenter, 1985; Adams *et al*., 1991), whereas one study claims it to be in the chloroplast thylakoid lumen (Spetea *et al*., 2004). The presence of many isoforms at different locations within the cell suggests that NDPK is an important enzyme with a fundamental function.

The primary function of NDPK is the maintainance of cellular homeostasis of NTPs and NDPs. It catalyses the synthesis of nucleoside triphosphates from nucleoside diphosphates through a ping-pong mechanism involving a phosphorylated enzyme intermediate (Parks and Agarwal, 1973). Its active site is made up of a fully conserved residue (histidine) that has been identified by direct protein sequencing (Gilles *et al*., 1991), site-directed mutagenesis, and X-ray crystallography (Dumas *et al*., 1992).The kinetic equations for the reaction have been reported by Garces and Cleland, 1969.

$$N_1TP + E\text{-}His \leftrightarrow N_1DP + E\text{-}His\text{-}P$$
$$N_2DP + E\text{-}His\text{-}P \leftrightarrow N_2TP + E\text{-}His$$

$$N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$$

For long, this regulation of the nucleoside triphosphate balance in a cell was known to be the main function of NDPKs. More recently, specific regulatory functions have also been attributed to them in addition to the catalytic function. The animal NDPKs have been reported to play key roles in processes such as cell differentiation (Okabe-Kado *et al*., 1988; Lakso *et al*., 1992), control of cell proliferation (Keim *et al*., 1992), motility (Kantor *et al*., 1993), development (Dearolf *et al*., 1988) and regulation of transcription (Postel *et al*., 1993). On the other hand, much less is known about the possible role of plant NDPKs.

The study of plant NDPKs started less than 15 years ago when the first plant genes homologous to NDPK were isolated from *Arabidopsis thaliana* (Quigley, 1992) and spinach (Nomura *et al*., 1992). Since then, NDPK genes have been cloned from several plant species. They have been shown to be involved in plant embryo development (Nato *et al*., 2000), seed development (Yano *et al*., 1995), and in response to blue light (Ogura *et al*., 1999) and high ionic strength (Kawasaki *et al*., 2001) but much remains unclear about their function in plant growth and development.

More recently a number of studies have been carried out to find the role of the NDPK2 isoforms in plant cells. Its role in signal transduction has come up as a major contribution, besides the housekeeping function. NDPK2 was shown to be a component of the UV-responsive pathway in *Arabidopsis thaliana* (Zimmermann *et al*., 1999). It was reported to be a component of the phytochrome signalling pathway in *Arabidopsis thaliana* (Choi *et al*., 1999) and to play a regulatory role in H_2O_2-mediated mitogen-activated protein kinase (MAPK) signalling in plants (Moon *et al*., 2003). The role of atNDPK2 in auxin-related cellular processes was demonstrated by Choi *et al*. (2005), where they showed that NDPK2 is involved in the regulation of auxin distribution.

Despite increasing evidence of the role of NDPK2 in various cellular processes and signal transduction pathways, there has not been an exhaustive study of its localisation within the plant cell. The published results from all the plants studied indicate the presence of NDPK2 isoform to be in chloroplasts, with the only exception of the NDPK2 of *Arabidopsis thaliana*. Two independent groups (Choi *et al*., 1999 and Zimmerman *et al*., 1999), through GFP-fusion experiments, have shown atNDPK2 to be localised in the cytosol and the nucleus. On the other hand this isoform has been purified from pea (Luebeck and Soll, 1996)

and spinach (Yang and Lampa, 1996) chloroplasts. These conflicting views led to the investigation, in the present study, of the exact localisation of atNDPK2 in a plant cell.

Out of all the plant NDPK2s isolated and characterised till date, the one from pea is of special interest because it was found to have two active forms in chloroplasts (Luebeck and Soll, 1995). Both the forms were shown to be translated from the same mRNA. In continuation of this previous study conducted in this laboratory, these two forms of psNDPK2 were further characterised. In addition, it was endeavoured to find out the reason for the occurrence of the two active forms of this isoform in chloroplasts.

To sum up, the aims of the present study are as follows:

1. To find out the sites of phosphorylation in the transit peptides of APC1, HCF136 and CAO.
2. To study the effect of this phosphorylation in transit peptides upon the import of preproteins into chloroplasts.
3. To biochemically characterise the two forms of psNDPK2 and study their import behaviours.
4. To establish the localisation of atNDPK2 within the plant cell.

4. Materials

4.1 Chemicals

All chemicals were purchased in highest available quality from Sigma Aldrich (Munich, Germany), Roth (Karlsruhe, Germany), Roche (Penzberg, Germany) and Merck (Darmstadt, Germany). Radio-labelled amino acids and nucleotides were obtained from Amersham Biosciences (Freiburg, Germany).

4.2 Enzymes and Kits

Restriction endonucleases were purchased from MBI Fermentas (St. Leon-Rot, Germany) and NEB (Boston, USA), T4 DNA ligase, RNA polymerase and Taq polymerase from MBI Fermentas and Eppendorf (Hamburg, Germany). DNase I and thermolysin were purchased from Roche (Penzberg, Germany), trypsin from Sigma Aldrich (Munich, Germany), RNase from Amersham Biosciences (Freiburg, Germany). Cellulase "Onozuka R-10" from *Trichoderma viride*, Macerozyme R-10 from *Rhizopus sp.* and lysozyme were supplied by Serva (Heidelberg, Germany).

Bacterial plasmid DNA isolation was done using the Plasmid Midi- or Maxi-Kit from Macherey and Nagel (Düren, Germany). Purification of DNA fragments from agarose gels was performed by the Nucleospin Extract II kit from Macherey and Nagel (Düren, Germany) and purification of PCR products was carried out using the QIAquick PCR purification Kit provided by Qiagen (Hilden, Germany). *In vitro* translation was done with wheat germ extract or reticulocyte lysate translation kit supplied by Promega (Madison, USA). Qiagen RNeasy Plant Mini kit was used for total RNA isolation from *Arabidopsis* plants. Dynabeads mRNA DIRECT kit from Dynal Biotech was used for the isolation of polyA mRNA from twenty-one days old *Arabidopsis thaliana* plants.

4.3 Molecular weight and size markers

Proteins weight standard MW-SDS-70L from Sigma was used for SDS-PAGE. DNA fragments size marker for agarose gels was prepared by restricting λ–phage DNA (MBI Fermentas) with EcoRI/HindIII restriction enzymes or with PstI enzyme.

4.4 DNA primers

The DNA primers used in PCR reactions were ordered from MWG-Biotech (Ebersberg, Germany), Qiagen (Hilden, Germany) and Invitrogen (Karlsruhe, Germany).

4.5 Bacterial strains

For DNA amplification, the *E.coli* strain DH5α (GibcoBRL, Eggenstein) was used whereas for the purpose of overexpression of proteins, BL21(DE3) cells (Novagen, Madison, USA) were used.

4.6 Plant material

Pea (*Pisum sativum*) seeds of the sort “Arvica” (Praha, Czech Rebublik) were used for the growth of plants. Wild type *Arabidopsis thaliana* seeds of the ecotype Columbia (Col-O) were obtained from Lehle, Round Rock, and TX 78680-2366, USA. Seeds of *Nicotiana tabacum* cv. petite Havana were used for the growth of tobacco plants.

4.7 Vectors

Constructs for overexpression and *in vitro* transcription and translation were cloned in pET21a vector (Novagen, Madison, USA) or pEXP-DEST2 (Gateway vector). For GFP and RFP fusion constructs, pOL-GFP and pOL-RFP were used, respectively.

4.8 Membranes and filters

Nitrocellulose-membrane (Protran BA-S83, 0.2 µm) was purchased from Schleicher and Schüll (Dassel, Germany). Sterile filters (Filtropurs 0.2, pore size 0.2 µm) were obtained from Sarstedt (Nuernberg, Germany) and 0.37 mm blotting-papers were from Schleicher and Schuell.

4.9 Antisera

Primary polyclonal antibodies (α-Tic110, α-Toc64, α-Tic32, α-Tic22, α-LHCP and α-atFNR-L1, α-pea14-3-3, α-APC1, α-CAO, α-HCF136) were raised in the laboratory of Prof. J. Soll. For raising pea α-NDPK2, the purified heterologously expressed full-length protein (see section 5.2.2) was sent to Biogenes GmbH, Berlin. Antibody against GFP, rabbit IgG fraction, from *Aequorea Victoria,* was purchased from Molecular Probes, USA, while purified mouse Anti-DsRed Monoclonal antibody was obtained from BD Pharmingen. Secondary antibodies against rabbit- and mouse-IgG conjugates from goat were purchased from Sigma (Munich, Germany).

4.10 Films and imaging plates

Kodak Biomax-MR films were bought from Eastman Kodak Company (Heidelberg, Germany). Fuji film imaging plates were used for imaging analysis. They were provided by Fuji photo film company, Japan.

4.11 Columns and column materials

The Ni-NTA Superflow column material was supplied by Qiagen (Hilden, Germany), and Talon affinity column material was from Clontech. Protein-A Sepharose was supplied by Amersham Biosciences (Freiburg, Germany). Micro Bio-Spin 6 Chromatography Columns (BioRad Laboratories, Hercules, CA) were used to deplete the translation product of ATP. Protein concentration columns (Centricon and Microcon 10K) were purchased from Millipore.

4.12 Equipment for Microinjection

The facilities for micro-injection of protoplasts were bought from Eppendorf (Hamburg, Germany). The equipment consisted of micromanipulators, CellTram® Air and Femtojet. The injection needles used were Femtotips (1.0 µm outer diameter and 0.5µm inner diameter) and Femtotips II (0.7 µm outer diameter and 0.5µm inner diameter) and the holding capillary was the VacuTip (outer diameter of 100µm and inner diameter of 15µm). Microloaders were used for filling the microinjection capillaries. The inverted microscope and the software used were from Till Photonics. 'Injection plates' were specially constructed for the purpose of microinjection by making a circular hole of 1.5 cm in diameter in the middle of the lid of a 35 x 10 mm Petri dish, followed by gluing a glass slip from underneath.

4.13 Bioimaging

Fluorescence images were obtained either by an epi-fluorescence microscope or Leica SP5 confocal microscope. The epifluorescence microscope was the polychrome IV System (Till Photonics GmbH, Munich, Germany) and GFP, rhodamin, DAPI and FITC filter sets were used. Pictures were taken with a cooled IR CCD camera and visualised by the TILLvision 4.0 software. The pictures were then given false colours using Adobe Photoshop 7.0 program.

5. Methods

5.1. Molecular biological methods

5.1.1 General molecular biological methods

Bacterial strain culturing, transformation with DNA, purification of restricted plasmid DNA by phenol/chloroform extraction followed by ethanol or isopropanol precipitation and preparation of glycerol stocks were performed according to the standard protocols (Sambrook *et al.*, 1989). Competent cells for DNA transformation were prepared according to Hanahan *et al.*, 1985.

5.1.2 Plasmid DNA isolation

Isolation of plasmid DNA from 3ml culture for restriction analysis was performed following the method of Birnboim and Doly (1979). For high purity and high yield DNA isolation, the Plasmid Midi- or Maxi- Kit from Macherey and Nagel (Düren, Germany) were used according to the manufacturer's instructions.

5.1.3 Cloning techniques

5.1.3.1 Polymerase chain reaction (PCR)

The restriction sites for cloning of DNA fragments into plasmid vectors were added by the polymerase chain reaction (PCR) (Saiki *et al.*, 1998). Standard PCR reactions included 100 ng DNA template, 200 µM of each dNTPs, 50 pmol of primers and 1-2 units of taq polymerase (Qiagen) or Triple Master taq polymerase (Eppendorf) in the supplied buffer. Temperatures were adjusted corresponding to the annealing temperatures of the primers. Recombinant PCR technique (Higuchi, 1990) was used to create the DNA coding for chimerical protein constructs. Fragments of the constructs were synthesized separately in standard PCR reactions, purified over agarose gels by QIAquick PCR Purification Kit and used as templates for the second round of PCR (20 ng of each fragment in the reaction) with the two outer primers, resulting in complete recombinant constructs (Sambrook *et al*., 1989).

5.1.3.2 General cloning methods

Isolation, restriction, ligation and agarose gel electrophoresis of DNA were performed according to standard procedures (Sambrook *et al.*, 1989). The reaction conditions for the enzymes were adjusted according to the protocols provided by the manufacturers.

5.1.3.3 Cloning Strategy

Primers with flanking restriction sites were used to introduce the required sequence of the pea and *Arabidopsis thaliana* proteins into the vectors. Point mutations were introduced by recombinant PCR.

The following oligonucleotides were used for the construction of the clones presented in Table 1.

APC1

APC1_SpeI	5'-ggactagtatggcttgctc–3'
APC1_KpnI	5'-ggggtaccaacctgtgcattag–3'
APC1_mSSU(+)	5'-cctccttcacgagcttccatgcaggtgtggccacc–3'
APC1_mSSU(-)	5'-ggtggccacacctgcatggaagctcgtgaaggagg–3'
APC1_16SA_for	5'-tgggtttcatcaaaaccagctctttctgctgattc–3'
APC1_16SA_rev	5'-tgaatcagcagaaagagctggttttgatgaaaccc–3'
APC1-18-SA(+)	5'- tcaaaaccatctcttgctgctgattcatcttcc –3'
APC1-18-SA(-)	5'-ggaagatgaatcagcagcaagagatggttttga–3'
APC1-25-SA(+)	5'-gattcatcttccttagcattccgatctgttctc–3'
APC1-25-SA(-)	5'-gagaacagatcggaatgctaaggaagatgaatc–3'
APC1-28-SA(+)	5'-ccttatcattccgagctgttctcaagtgc–3'
APC1-28-SA(-)	5'-gcacttgagaacagctcggaatgataagg–3'
APC1_37/38S/A_for	5'-ctaacactgccgcacctccttc–3'
APC1_37/38S/A_rev	5'-gaaggaggtgcggcagtgttag–3'
APC1_41SA_for	5'-aacacttcctcacctcctgcacgagcttcctctg–3'
APC1_41SA_rev	5'-aacagaggaagctcgtgcaggaggtgaggaagtg–3'
APC1_45/46SA_for	5'-tcacctccttcacgagctgccgctgtttcaccactc–3'
APC1_45/46_rev	5'-gagtggtgaaacagcggcagctcgtgaaggaggtg–3'
APC1_47S/A_for	5'-cctctgttgcaccactccaagc–3'
APC1_47S/A_rev	5'-gcttggagtggtgcaacagagg–3'
APC_25ST_for	5'-tcttccttaacattccgatctg–3'
APC_25ST_rev	5'-agatcggaatgttaaggaagatg–3'
Apc_d_13	5'-gggaattccatatgaaaccatctctttctgct g–3'
Apc_d_23	5'-gggaattccatatgttatcattccgatctgttctc–3'
Apc_d_47	5'-gggaattccatatgccactccaagcgtctcttcg–3'
mSSU_ndeI	5'-gggaattccatatgcaggtgtggccaccaattaac–3'

HCF136

Hcf136_G_for	5'-aaaaagcaggcttagaaggagatagaaccatggcgtctctgcaactctgc–3'
Hcf136_G_rev	5'-agaaagctgggtcgtagccttctggcttgtaggc–3'
Hcf_mSSUf	5'- ggagcttctgtaccaatcgatgcaggtgtggccacc–3'
mSSU_Hcfr	5'- ggtggccacacctgcatcgattggtacagaagctcc–3'
Hcf_d18_mSSU_for	5'-gaaggagatagaaccatgcctcgattcctc–3'
Hcf_d18_mSSU_rev	5'-gaggaatcgaggcatggttctatctccttc–3'
Hcf_d37_mSSU_for	5'-gaaggagatagaaccatgcctcctccatctc–3'
Hcf_d37_mSSU_rev	5'-agatggaggaggcatggttctatctccttc–3'
Hcf_52/54_ST_for	5'-tcatccttaactttcactcggcg–3'
Hcf_52/54_ST_rev	5'-ccgccgagtgaaagttaaggatg–3'

CAO

CAO_NotI	5'-gcccaaaaggggcggccgcatgaacgccgccgtgtttag–3'
CAO_XhoI	5'-cccgggctcgaggccggagaaaggtagtttatc–3'
CAO _NdeI	5'-ggcatatgaacgccgccgtgtttag-3'
pCAO_NsiI	5'-cctccacaatgcataacggtctccattg-3'

LHCP

LHCP_ndeI	5'-gggaattccatatggccgcatcatc–3'
LHCP_xhoI	5'-taagagctcgagttttccggg–3'
LHCP_d41_for	5'-gaaggagatagaaccatgaggaagtctgctac–3'
LHCP_d41_rev	5'-catggttctatctccttctaagc–3'
LHCP_d8_for	5'-gaaggagatagaaccatggctctctcttctc–3'
LHCP_d8_rev	5'-agaagagagagccatggttctatctccttc–3'
LHCPd8_1213ST_for	5'-gaaggagatagaaccatggctctcactactcca ac–3
LHCPd8_1213ST_rev	5'-tggagtagtgagagccatggttctatctccttc–3'
LHCP_1213_ST_f	5'-atggctctcactactccaaccttg–3'
LHCP_1213_ST_r	5'-caaggttggagtagtgagagccat–3'
LHCP_G_for	5'-aaaaagcaggcttagaaggagatagaaccatggccgcatcatcatcatc–3'

LHCP_G_rev	5'-agaaagctgggtcttttccgggaacaaagttggt–3'
LHCP_471	5'-gctcaaagcatccttgccatatgg–3'
pLHCP_NdeI	5'-taagaaggagatatacatatgaacggattctagaattgc –3'
pLHCP_XhoI	5'-gtggtggtgctcgagttttccggg –3'
LHCP_KpnI	5'-ggggtaccgttttccgggaac–3'

pSSU#mLHCP

pLHCP_mSSU_for	5'-ggagctgcaaggttcaccatgcaggtgtggccacc–3'
pLHCP_mSSU_rev	5'-ggtggccacacctgcatggtgaaccttgcagctcc–3'
pSSU_LHCP_for	5'-ggcggaagagtgcaatgcatgaggaagtctgc–3'
pSSU_LHCP_rev	5'-gcagacttcctcatgcattgcactcttccgcc–3'

pSSU

pSSU_NdeI	5'-cccgggcatatggcttcctcagttctttcc–3'
pSSU_XhoI	5'-cccgggctcgaggtagccttctggcttgtagg–3'
mSSU_HindIII	5'-ggaagagtgaagcttatgcaggtgtg–3'
mSSU_ClaI	5'-ggaagagtgatcgatatgcaggtgtg–3'
pSSU_KpnI	5'-ggggtaccggtagccttctg–3'
pSSU_GFP_for	5'-ggactagtatggcttcctc–3'
pSSU_GFP_rev	5'-ggggtaccgtagccttctg–3'

Primers for Gateway System Cloning

attB1adapter	5'-ggggacaagtttgtacaaaaaagcaggct–3'
attB2adapter	5'-ggggaccactttgtacaagaaagctgggt–3'

Table 1. The constructs prepared for the study of the sites of phosphorylation are listed. Given are the name (column 1), the vector used (column 2), biological source (column 3) and the purpose for cloning (column 4) of the constructs.

Construct	Vector	Organism	Purpose
pCAO#mSSU	pET21a	*Arabidopsis thaliana* (pCAO) *Nicotiana tabacum (*mSSU)	Overexpression *In vitro* phosphorylation
pAPC1#mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1_16S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1-18S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-25S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1-28S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation

pAPC1-37/38S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-41S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-44/45 S/A#mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1-47S/A #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-d13# mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1-d23# mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pAPC1-d47# mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-d75# mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pAPC1-25-S/T #mSSU	pET21a	*Arabidopsis thaliana* (APC1) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
HCF136-pDONR201	pDONR201	*Arabidopsis thaliana*	Overexpression *In vitro* phosphorylation
HCF136-pEXP2_DEST	pEXP_ DEST	*Arabidopsis thaliana*	Overexpression *In vitro* phosphorylation
pHCF136# mSSU-pEXP2 _DEST	pEXP2_ DEST	*Arabidopsis thaliana* (HCF136) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pHCF136-d18 #mSSU-pEXP2_DEST	pEXP2_ DEST	*Arabidopsis thaliana* (HCF136*)* *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pHCF136-d37 #mSSU-pEXP2_DEST	pEXP2_ DEST	*Arabidopsis thaliana* (HCF136*)* *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pHCF136-52/54 S/T#mSSU-pEXP2_DEST	pEXP2_ DEST	*Arabidopsis thaliana* (HCF136*)* *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation

pHCF136-d37 #mSSU-pEXP2_DEST	pEXP2_ DEST	*Arabidopsis thaliana* (HCF136) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
LHCP-p DONR201	pDONR201	*Pisum sativum*	Overexpression *In vitro* phosphorylation
LHCP-pEXP2 _DEST	pEXP_ DEST	*Pisum sativum*	Overexpression *In vitro* phosphorylation
LHCP-26/27-S/A-pEXP2 _DEST	pEXP2_ DEST	*Pisum sativum*	Overexpression *In vitro* phosphorylation
LHCP-d41-pEXP2 _DEST	pEXP2_ DEST	*Pisum sativum*	Overexpression *In vitro* phosphorylation
LHCP-d8-pEXP2_DEST	pEXP2_ DEST	*Pisum sativum*	Overexpression *In vitro* phosphorylation
LHCP-d8-12/13-S/T -pEXP2_DEST	pEXP2_ DEST	*Pisum sativum*	Overexpression *In vitro* phosphorylation
pLHCP#mSSU pEXP2_DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pLHCP-d8 #mSSU pEXP2_DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pLHCP-d8-12/ 13-S/T#mSSU pEXP2_DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pLHCP-26/27-S/A#mSSU pEXP2_DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum (mSSU)*	Overexpression *In vitro* phosphorylation
pSSU-pEXP2 _DEST	pEXP2_ DEST	*Nicotiana tabacum*	Overexpression *In vitro* phosphorylation
pSSU-31/34-S/A-pEXP2 _DEST	pEXP2_ DEST	*Nicotiana tabacum*	Overexpression *In vitro* phosphorylation
pSSU-GFP-pOL	pPOL-GFP	*Nicotiana tabacum*	Transient expression in protoplasts
pSSU-31/34SA-GFP-pOL	pPOL-GFP	*Nicotiana tabacum*	Transient expression in protoplasts
pSSU#LHCP pEXP2 _DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum* (mSSU)	Overexpression *In vitro* phosphorylation
pSSU-31/34-S/A# LHCP-pEXP2 _DEST	pEXP2_ DEST	*Pisum sativum* (LHCP) *Nicotiana tabacum(pSSU)*	Overexpression *In vitro* phosphorylation

T7P-LHCP-GFP-pPOL	pPOL-GFP	*Pisum sativum*	Transient expression in protoplasts
T7P-pSSU#LHCP-GFP-pPOL	pPOL-GFP	*Pisum sativum* (LHCP) *Nicotiana tabacum*(pSSU)	Transient expression in protoplasts
T7P-pSSU-31/34-S/A#LHCP-RFP-pPOL	pPOL-RFP	*Pisum sativum* (LHCP) *Nicotiana tabacum(pSSU)*	Transient expression in protoplasts
T7P-pSSU-31/34-S/A-LHCP-GFP-pPOL	pPOL-GFP	*Pisum sativum* (LHCP) *Nicotiana tabacum(pSSU)*	Transient expression in protoplasts

Table 2. Other constructs used in the present study, which were a kind gift from Prof. Colin Robinson, University of Warwick, UK are as follows:

Construct	Vector	Organism	Purpose
pOE23-GFP-pDHA	pDHA	*Pisum sativum*	*In vivo* labelling
pOE23-22SA-GFP-pDHA	pDHA	*Pisum sativum*	*In vivo* labelling
OE23-pDHA	pDHA	*Pisum sativum*	*In vivo* labelling
OE23-22SA-pDHA	pDHA	*Pisum sativum*	*In vivo* labelling

Primers for cloning the pea NDPK2 constructs:

NDPK_NheI_for	5'- ctagctagcatggaagccgtg -3'
NDPK_KPNI_rev	5'- cggggtaccgttctcttagccatg -3'
Ndpk_63RD_for	5'- ttccgcaccaacagcagcacc -3'
Ndpk_63RD_rev	5'- cggtgctgctgttggtgcggaa -3'
NDPK_74RD_f	5'- ttccttcccgacctaatcacttctctcgaa -3'
NDPK_74RD_r	5'- ttcgagagaagtgattaggtcgggaaggtt -3'
Ndpk77AT_for	5'- cgcctaatcacttctctcgaac -3'
Ndpk77AT_rev	5'- ttcgagagagaagtgattaggcg -3'
N_112KA_for	5'- gagaaaaaggggtttgcgttaactggcttg –3'
N_112KA_rev	5'- caagccagttaaccgcaaaccccttttttctc –3'
N_213KH_for	5'- gctctatggttccacgaaggcgaattatgcg –3'
N_213KH_rev	5'- cgcataattgcgcttcgtggaaccatagagc –3'

Primers for cloning the *Arabidopsis thaliana* NDPK2 constructs:

N2a_Ara_NheI_for	5'- ctagctagcatggtgggagcg –3'
N2a_Ara_KPNI_rev	5'- cggggtaccgctcccttagcc – 3'
NIa-79_NheI	5'-ctagctagcatggaggacgttgag g-3'

Gene specific primers for RT-PCR:

NA_for1	5'- ctgagagaagctaagacaaagc – 3'
NA_rev1	5'- gccaattcttttgggcactgaaac – 3'
NA_for2_nested	5'- cagggaaagagaaagagtctg – 3'
NA_rev2_nested	5'- ccccttttctcaaagcgag – 3'

Gene specific primer for RACE-PCR:

N_Ara_Ex7_rev 5' -gctagagccgaatcccacttgcatagc – 3'

Table 3. The constructs prepared for the NDPK2 study are listed. Given are the name (column 1), the vector used (column 2), biological source (column 3) and the purpose for cloning (column 4) of the constructs.

Construct	Vector	Organism	Purpose
psNDPK2-pET21a	pET21a	*Pisum sativum*	*Transcription/Translation*
psNDPK2-63RD-pET21a	pET21a	*Pisum sativum*	*Transcription/Translation*
psNDPK2-74RD-pET21a	pET21a	*Pisum sativum*	*Transcription/Translation*
psNDPK2-77AT-pET21a	pET21a	*Pisum sativum*	*Transcription/Translation*
psNDPK2-RFP	pOL-RFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-63RD-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-74RD-RFP	pOL-RFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-74RD-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-77TA-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
psNDPK2-112KA-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
psNDPK-213KH-GFP	pOL-GFP	*Pisum sativum*	Protoplast Transformation
atNDPK2-GFP	pOL-GFP	*Arabidopsis thaliana*	Protoplast Transformation
atNDPK2-Δ1-79-RFP	pOL-RFP	*Arabidopsis thaliana*	Protoplast transformation

Other constructs used in the study of the NDPK are as follows:
atNDPK2-pBluescript construct was bought from Riken, Japan.
APO1-GFP-pOL was a kind gift from Dr. Joerg Meurer, LMU, Munich.

5.1.4 RT- and RACE-PCR

The RT-PCR (Invitrogen, Karlsruhe, Germany) and RACE-PCR (BD SMART RACE cDNA Amplification kit, Clontech, US) were performed according to the manufacturer's recommendation.

5.1.5 Isolation and purification of chloroplast DNA

Chloroplasts from pea plants were isolated as described in section 5.3.1. Chloroplasts were pelleted and resuspended in 600 µl Extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl; 2% SDS and ß-mercaptoethanol added just before use) incubated for 20 minutes at 65°C with shaking. To this 200 µl 3 M Kac were added, mixed and kept on ice for 15 minutes. This was centrifuged at 15,500 g for 10 minutes at 4°C. The supernatant was taken in a new Eppendorf tube and the centrifugation step was repeated. 470 µl of isopropanol (-20°C) were added to the supernatant, incubated at room temperature for 30 minutes and centrifuged at 15,500 g for 15 minutes at 4°C. The pellet was washed with 500

µl 75% ethanol (-20°C) and dried. The pellet was suspended in 100 µl water and 1µl RNase A was added to it. This was incubated for 30 minutes at 37°C. It was followed by phenol-chloroform extraction and precipitation with 2 M sodium acetate. After washing with 75% ethanol, the pellet obtained was resuspended in 50 µl water and it contained the genomic DNA. Various restriction controls were done to check for genomic contamination (Palmer and Thompson, 1981).

5.2. Biochemical methods

5.2.1. General biochemical methods

Protein concentrations were determined by using the Biorad Protein Assay reagent (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer's recommendations. Precipitation of proteins was performed by using tri-chloroacetic acid (TCA) according to published procedures (Sambrook *et al.*, 1989). SDS-PAGE was performed according to Laemmli (1970). All the gels used in the present study were either 12.5% or 15% gels. Gels were stained either with Coomassie Brilliant Blue R250 or silver-stained as described by Blum *et al.*, 1987. For immunodetection, proteins were transferred onto a nitrocellulose membrane by semi-dry blotting (Towbin *et al.*, 1979). Immunodecoration and detection was done by using the alkaline phosphatase system as described (Sambrook *et al.*, 1989).

For the detection of ^{35}S-labelled proteins, the acrylamide gels were stained with Coomassie Blue, fixed, and after drying, they were laid on phosphor-imaging plates (BAS-MS, Fuji Photo Film Co. Ltd, Japan) for 2-16 hours. The plates were screened using phosphoimage scanner FLA-3000 and band intensities were analyzed using AIDA program for advanced image analysis.

5.2.2 Overexpression of recombinant proteins and preparation of inclusion bodies

Expression of all proteins under study was performed in *E. coli* strain BL21(DE3) using the pET21a vector (Novagen, Madison, USA) or the pEXP-DEST vector, both containing a 6x-His tag. The transformed cells were grown at 37°C in 200 ml M9ZB medium to OD_{600}=0.5-0.6 and the expression was initiated by addition of 1mM IPTG. After two hours of incubation at 37°C, the cells were harvested by centrifugation and the expressed protein was isolated in the form of inclusion bodies. The pellet containing the bacterial cells was resuspended in Lysis buffer (50 mM Tris/Cl pH 8.0, 25% sucrose, 1 mM EDTA) and disrupted in a French Press cell at 1200 bar (18,000-20,000 psi). The resulting suspension was sonicated 2 times for 10 seconds to shear the genomic DNA, and centrifuged at 15,000 g for 30 minutes to pellet the unsoluble membrane material. The resulting pellet was resuspended in Detergent buffer (20 mM Tris/Cl pH 7.5, 1% Desoxycholic acid, 1% Nonidet P40, 200 mM NaCl, 2 mM EDTA, 1 mM Mercaptoethanol) and centrifuged for 10 minutes at 9000 g. The pellet thus

obtained was washed 2 times with Triton buffer (20 mM Tris/Cl pH 7.5, 0.5% Triton X-100, 2mM EDTA, 1 mM Mercaptoethanol) and 2 times in Tris buffer (50 mM Tris/Cl pH 8.0, 1 mM EDTA, 10 mM DTT). The inclusion bodies were finally resuspended in Tris buffer and subsequently frozen in liquid nitrogen and stored at -80°C.

5.2.3 Purification of proteins by affinity chromatography

The inclusion bodies were denatured in Buffer A (8 M Urea, 200 mM NaCl and 20 mM Tris/Cl pH 8.0), centrifuged at 20,000 g for 10 minutes and the supernatant containing the soluble protein was incubated with pre-equilibriated Ni-NTA agarose column for 1 hour at room temperature. The column was then washed extensively with Buffer A. The elution of the protein was performed with Buffer E (Buffer A + 150 mM Imidazol)

5.2.4 *In vitro* transcription and translation

In vitro transcription of linearized plasmids was carried out in a reaction volume of 50 µl containing transcription buffer (supplied by the manufacturer), 10 mM DTT, 100U RNase inhibitor, 0.05% (w/v) BSA, 0.5 mM ATP, CTP, and UTP, 0,375 mM m7-Guanosin (5') ppp (5') Guanosin (cap), 10U T7 RNA polymerase and 2-2.5 µg plasmid DNA. The reaction mixture was incubated for 15 minutes at 37˚C to yield RNA with a cap at the 5' end. Finally, 12 mM GTP was added and the transcription mixture was incubated for another 60-90 minutes. The mRNA obtained was either used directly for *in vitro* translation or stored at -80°C. *In vitro* translation of mRNA was carried out using reticulocyte lysate or wheat germ translation kit (Promega), following the manufacturer's instructions, with optimal RNA concentration and adjusted potassium acetate and magnesium acetate, which were determined by test translation. 150 µCi of [^{35}S]-methionine/cysteine mixture were added for radioactive labelling.

5.2.5 Phosphorylation assay and Phospho-amino acid analysis

The phosphorylation reactions and phospho-amino acid analysis were performed exactly as described in Waegemann and Soll (1996).

5.2.6 Immuno-precipitation under denaturating conditions after phosphorylation of proteins

Two phosphorylation reactions were carried out and the reaction was stopped with 2% end concentration of SDS. It was diluted to 0.2% with IP buffer I (25 mM Tris pH 7, 150 mM NaCl.) 5 µl of antibody was added to the reaction which was then rotated for 1h at room temperature. In the meantime Protein A Sepharose was equilibrated with IP buffer II (25 mM Tris pH 7, 150 mM NaCl, 0.05% Egg-Albumin) and then with IP buffer III (25 mM Tris pH 7, 150 mM NaCl, 0.2% SDS) once. The sample was added to the equilibrated Protein

Sepharose A and allowed to rotate for another hour at room temperature. It was then washed twice with IP buffer III and with IP buffer I. The elution was done with 30 µl 2x Laemmli buffer which was vortexed for 45 seconds and then heated for 45 seconds at 95°C. The elution step was repeated again and loaded onto the gel along with 1% samples from all the above steps.

5.3 Cell biological methods

5.3.1 Isolation of intact chloroplasts from pea

The isolation of intact chloroplasts from 12 days old pea seedlings grown under 12/12 hours dark/light cycle was performed as described by Waegemann and Soll (1991). The determination of chlorophyll concentrations was carried out as described by Arnon (1949).

5.3.2 ATP depletion from isolated chloroplasts and *in vitro* translation product

Intact chloroplasts were left on ice in the dark for 30 minutes in order to deplete ATP. Micro Bio-Spin 6 Chromatography columns were used to remove the ATP from the *in vitro* translation products.

5.3.3 Import of radioactively-labeled proteins into intact chloroplasts

A standard import reaction contained chloroplasts equivalent to 20 µg chlorophyll in 100 µl import buffer (10 mM methionine, 10 mM cysteine, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 2% BSA (w/v), 330 mM sorbitol, 50 mM HEPES-KOH, pH 7.6, 5 mM $MgCl_2$) and 1-5% *in vitro* translation product. Import reactions were initiated by the addition of translation product and carried out for 15 minutes at 25°C unless indicated otherwise. Reactions were terminated by separation of chloroplasts from the reaction mixture by centrifugation through a 40% (v/v) Percoll cushion in import buffer. Chloroplasts were washed once and import products separated by SDS-PAGE and radiolabelled proteins analysed by a phosphor-imager.

5.3.4 Protease treatment of intact chloroplasts after import

After import the translocation of proteins through the outer chloroplast envelope was controlled by the treatment of intact organelles with thermolysin. Chloroplasts were pelleted from the import reaction at 1,500 g for 1 minute at 4°C, resuspended in 100 µl digestion buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6, 0.5 mM $CaCl_2$). The reaction was started with the addition of thermolysin (200 µg/mg chlorophyll) and was incubated on ice for 20 minutes. The proteolysis was stopped by addition of EDTA to 10 mM end concentration; chloroplasts were pelleted again and washed in the digestion buffer with addition of 5 mM EDTA. For trypsin digestion, the purified chloroplasts were incubated with 0.5-1 µg trypsin per µg of chlorophyll in 330 mM sorbitol, 50 mM Hepes/KOH pH 7.6, 500 µM $CaCl_2$ at 25°C

for 1-30 minutes. The reactions were stopped by addition of ten fold excess of trypsin inhibitor.

5.3.5 Chloroplast fractionation into soluble and insoluble fractions

To distinguish between integral membrane proteins and soluble or peripheral membrane proteins, the re-isolated chloroplasts were lysed in low osmotic urea buffered solution (6 M Urea, 10 mM Hepes/KOH pH 7.6) for 30 minutes on ice, followed by centrifugation at 100,000 g for 10 minutes at 4˚C to separate membranes from the soluble fraction. Membrane-integrated proteins cannot be solubilized from membranes under these conditions (Molloy *et al.*, 1998).

5.3.6 Chloroplast fractionation into their suborganellar compartments

For fractionation of chloroplasts after import, the re-isolated chloroplasts were resuspended in 1 ml buffer (0.65 M Sucrose, 10 mM Tricine pH 7.9, 1mM EDTA). The sample was frozen and thawed slowly 2 times and subsequently it was mechanically lysed and disrupted with a glass dounce-homogenizer and diluted to 0.3 M sucrose using phosphate buffer (10 mM NaPi pH 7.9, 1 mM EDTA) to a final volume of 2 ml. Chloroplasts were then applied on a sucrose gradient (0.465 M, 0.8 M and 0.996 M sucrose in 10 mM Tricine/KOH pH 7.9, 1 mM EDTA) and centrifuged at 250,000 g for 3 hours, in order to separate chloroplast compartments (outer envelope, inner envelope, stroma and thylakoids). Chloroplast membranes were washed with 10 mM NaPi buffer and centrifuged for 1 hour at 66,700 g. Thylakoids were washed three times with 10 mM NaPi buffer, after each wash samples were centrifuged for 1 minute at 1500 g. Soluble proteins of the stroma were TCA precipitated. The different fractions were subjected to SDS-PAGE.

5.3.7 Isolation of intact chloroplasts from *Arabidopsis thaliana* leaves

Arabidopsis thaliana chloroplasts were isolated from the leaves of 3-4 week old plants. The leaves were ground in isolation buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.33 M sorbitol, 14.3 mM β-mercaptoethanol) and the suspension was filtered through 25 µm gauze. Chloroplasts were collected after centrifugation (3 min at 1000 g, 4°C) and purified on a 30%-82% Percoll gradient (1500 g for 15 min, 4°C). Intact chloroplasts were collected from the 82% interface and washed twice with isolation buffer.

5.3.8 Isolation of chloroplasts from *Arabidopsis thaliana* protoplasts

Approximately two million protoplasts were suspended in 2 ml isolation buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH, 10 mM $NaHCO_3$, 50 mM ascorbate, pH 8), filtered through 25 µm gauze and centrifuged at 1000 g, 4°C for 5 minutes.

The pellet was resuspended in 1ml isolation buffer and layered onto Percoll gradients (lower-87%, upper-29%). This was centrifuged for 10 minutes, at 4°C, 1500 g, without brake in a swing-out rotor. The band between the two Percoll layers consisted of intact chloroplasts and was collected carefully. They were suspended in 5 ml HMS buffer (50 mM Hepes, 3 mM $MgSO_4$, 0.3M sorbitol, 50 mM ascorbate, pH 8) and centrifuged at 1000 g, 4°C for 5 minutes. The pellet was washed again with HMS and the pellet was resuspended in 200 µl HMS. The chloroplast density was quantified as for the pea chloroplasts (section 5.3.1).

5.3.9 Isolation of protoplasts from *Nicotiana tabacum* leaves

The isolation of protoplasts from *Nicotiana tabacum* leaves and the transformation with GFP or RFP fusion constructs was carried out as described in Dovzhenko *et al.* (1998). 3-4 week old tobacco plants grown on B5-modified medium (Gamborg *et al.*, 1976) were used. 1g leaves were cut with a razor blade into 0.1 cm wide stripes and incubated with 0.5% cellulase and 0.5% macerase in 10ml F-PIN medium (MS medium , PC-vitamins (200 mg/l Myo-inositol, 1.0 mg/l thiamin-HCl, 2.0 mg/l Ca-panthotenate, 2.0 mg/l nicotinic acid, 2.0 mg/l pyridoxin-HCl, 0.02 mg/l biotin), 1.0 mg/l 6-benzylaminopurin (BAP), 0.1 mg/l α-naphtaleneacetic acid (NAA), 20 mM MES, pH 5.8 (KOH), 80 g/l glucose; osmolarity-550 mOsm was adjusted with glucose) for 12-14 h. The resulting suspension was filtered through 100 µM nylon gauze in a 15 ml Sarstedt tube. 2 ml F-PCN medium (F-PIN, except instead of glucose, sucrose was added as the osmoticum) was overlayed on the filtered suspension and centrifuged for 10 minutes at 70 g without brake. The intact protoplasts were collected from the interface between the F-PIN and F-PCN media, washed with transformation medium and sedimented at 50 g for 10 min. Protoplasts were counted in a Fuchs-Rosenthal counting chamber and cell density was adjusted to $5x10^6$ protoplasts/ml in transformation medium (15 mM $MgCl_2$, 0.1% g/v MES, 0.5 M mannitol). The intactness of the protoplasts was controlled by observation bright–field microscopy.

5.3.10 PEG Transformation of *Nicotiana tabacum* protoplasts

40 µg of DNA were used for one transformation. In case of co-transformation, 20 µg of each DNA were used. 25 µl of DNA were added to 100 µl of protoplasts in a 35 mm Petri dish and gently mixed by shaking. 125 µl of 40% PEG 4000 were added, gently mixed and incubated for 7.5 min. PEG is a tensioactive material that helps the DNA to adhere to the plasma membrane. It, along with the permeabilising contribution of the calcium ions present in the transformation medium, makes DNA-uptake by the protoplasts more efficient. This was followed by the addition of 125 µl of F-PCN medium and further incubation for two minutes. The protoplast suspension was diluted with 2.5 ml of F-PCN medium and incubated in the

dark at 25°C. Specific GFP- and RFP-fluorescence could be observed after 15-17 hours after the transformation.

5.3.11 Isolation and PEG Transformation of *Arabidopsis thaliana* protoplasts

The protocol for isolating and transforming *Arabidopsis thaliana* protoplasts was adapted from Sheen, J. 2002, A transient expression assay using *Arabidopsis* Mesophyll protoplasts. http://genetics.mgh.harvard.edu/sheenweb/ .

5.3.12 Isolation of pea protoplasts

The isolation of pea protoplasts was performed as described in Glimelius, 1984. Briefly, leaves from 12 days old plants were plasmolysed for 1 h in 0.3 M sorbitol and 0.05 M $CaCl_2$. The digestion was performed in 1% Cellulase and 0.1% Macerase in modified K3 (according to Nagy and Maliga, 1976) containing 0.4 M sucrose, for 16-18 hours at 20-22°C in the dark. The protoplasts were filtered through a nylon mesh of 100 µm and salt solution (16% sucrose, KH_2PO_4- 27.2 mg/L, KNO_3 - 101 mg/L, $CaCl_2.2H_2O$ – 148 mg/L, $MgSO_4.7H_2O$-246.0 mg/L, KI- 0.16 mg/L, $SO_4.5H_2O$ - 0.025 mg/L) was added. This was centrifuged for 7 minutes at 100 g, without brake. The floating layer was taken with a Pasteur pipette and washed with W5 (Menczel *et al.* 1981) and centrifuged for 5 minutes at 75 g without brake.

5.3.13 PEG Transformation of *Pisum sativum* protoplasts

PEG transformation of pea protoplasts was performed as described in Negrutiu *et al.*, 1987.

5.3.14 Isolation of cytoplasm from pea and *Arabidopsis thaliana* protoplasts

Protoplasts were isolated as described in section 5.3.9. Around two million protoplasts were taken in 2 ml transformation medium and broken by a syringe with a 600 µm needle attached to it. The broken protoplasts were centrifuged for 5 minutes at 1200 g at 4°C. The pellet was resuspended in 2 ml 10 mM Hepes pH 7.6. The supernatant was further centrifuged in an ultra centrifuge at 300,000 g, for 3 hours at 4°C. The supernatant was collected as the cytoplasmic fraction.

5.3.15 Preparation of *Arabidopsis* Leaf Extract

4-5 leaves were taken in an Eppendorf tube and put immediately in liquid nitrogen. The Polytron homogeniser was pre-cooled by putting a beaker containing liquid nitrogen under it for some time. The leaves were then ground in an Eppendorf tube and 300 µl extraction buffer (40 mM Hepes/KOH pH 7.6, 100 mM KAc, 1 mM MgAc, 4 mM DTT, adjust pH to 7.6) were added to it followed by its centrifugation for 30 minutes at 150,000 g, 4°C.

5.3.16 Embedding of protoplasts in alginic acid

The embedding of protoplasts in alginic acid was done in accordance with Dovzhenko *et al.*, 1998. Briefly, alginic acid solution was prepared by mixing 120 mg of alginic acid in 10 ml of MMM buffer ($MgCl_2.6H_2O$-255 mg/250 ml, $MgSO_4.7H_2O$-312.5 mg/250 ml, mannitol 21.25 g/250 ml, MES-488 mg/250 ml, adjusted pH to 5.8 and osmolarity to 550 Osm). 500 µl of protoplast suspension were mixed with 500 µl alginic acid solution and put as a very thin layer on Ca-A plate ($CaCl_2.2H_2O$- 735 mg/250 ml, mannitol-21.25 g/250 ml, 10 mM MES, 1% Agar, pH 5.8) and allowed to solidify for 1 hour at room temperature. The alginate layer was scrapped gently using a scalpel and placed on the Petri plate which was filled with MMM buffer to surround the protoplast-containing layer.

5.3.17 Embedding of protoplasts in Agarose

The gel matrix was prepared by adding 3% (w/v) ultralow melting point agarose (GibcoBRL) to Gamborg's B5 medium supplemented with 0.6 M mannitol and heated in a microwave. After cooling at room temperature for 5-10 minutes, 40 µl were put on a cover slip and 2 µl of protoplast suspension were mixed in it. Another cover slip was put on top of this gel matrix and allowed to spread for 10 seconds to form an even layer. This was then incubated in a moist chamber for 1 hour at room temperature, followed by the removal of the upper coverslip and addition of 200 µl B5/mannitol solution to prevent the drying of the gel (Gilroy, 1996).

5.3.18 Attachment of protoplasts using Poly-L-Lysine

0.1% stock solution of poly-L-lysine was prepared in sterile water. A drop of 1:400 dilution of the stock solution was put on a coverslip and allowed to stand for 10 minutes. The cover slip was then washed with sea water (9.3 mM $CaCl_2$, 25.5 mM $MgSO_4$, 23 mM $MgCl_2$, 2 mM $NaHCO_3$, 9 mM KCl, 425 mM NaCl, pH 8.0) and a drop of protoplast suspension was put on it. It was allowed to stand for 5 min and then washed by adding K3 medium.

5.3.19 Staining of PEG transformed protoplasts with DAPI

4'-6-Diamidino-2-phenylindole (DAPI) is a substance which is known to form fluorescent complexes with natural double-stranded DNA. The transformed protoplasts were fixed with 2% paraformaldehyde (PFA) in PBS (8 g NaCl; 0.2 g KCl; 1.44 g $Na_2HPO_4 \cdot 7H_20$; 0.24 g KH_2PO_4 ; H_20 to 1 liter; pH 7.4) for 5 minutes, washed once with PBS and then permeabilized with 0.2% TritonX-100 for 5 minutes, followed by washing 3 times with PBS. They were then stained with 1 µg/ml final concentration of DAPI (4',6-Diamidino-2-phenylindolehydrochloride) for 10 minutes, washed 3 times with PBS and observed under the microscope.

5.3.20 *In vivo* labelling technique

5.3.20.1 Isolation and PEG transformation of tobacco protoplasts for *in vivo* labelling

10x enzyme mix (Macerozyme Onozuka R-10, 2% (w/v); Cellulase Onozuka R-10, 4% (w/v)) was prepared in K3 (Gamborg's B5 basal medium with minimal organics, 3.78 g/l; $CaCl_2.2H_2O$, 750 mg/l; NH_4NO_3, 250 mg/l; sucrose, 136.2 g/l; xylose, 250 mg/l; 6-BAP, 1 mg/l; a-naphtalenacetic acid (NAA), 1 mg/l) and stored at –20°C. It was diluted to a final concentration of 1x with K3 immediately before use and poured into 10 cm Petri dishes in 7 ml aliquots. 4-6 week old green leaves were cut from a sterile plant and carefully scarified on their underside. The mid-ribs were removed and the scarified leaves were transferred such that the wounded face was in contact with the enzyme mix. This was incubated in the dark at 26°C. Next morning the digestion mix was carefully removed using a sterile Pasteur pipette, leaving protoplasts still attached to the leaves. 3 ml K3 was added drop wise over the leaves to release the protoplasts which were then filtered through 100 µm nylon gauze. A further 3 ml K3 was added to the leaves to release the rest of the protoplasts which were also filtered. They were then centrifuged in 50 ml sterile Sarstedt tubes at 100 g for 20 minutes at room temperature, without brake. The pellet of broken protoplasts and most of the K3 above it was removed leaving 5 ml K3 containing the floating layer of viable protoplasts. Four volumes of W5 solution (NaCl, 9 g/l; KCl, 0.37 g/l; $CaCl_2.2H_2O$, 18.37 g/l; glucose, 0.9 g/l) was added dropwise down the wall of the tube. The solution was gently mixed until the protoplasts were evenly distributed. Protoplasts were pelleted by centrifugation at 100 g for 10 minutes at RT and the supernatant was aspired off. The resulting pellet was gently resuspended after the dropwise addition of W5 to the same end volume as in the previous step. The protoplasts were carefully resuspended in upto 10 ml W5 and incubated in the dark for 30 minutes at RT, after taking a sample for counting.

After incubation in the dark, the protoplasts were pelleted by centrifugation at 100 g for 5 min at room temperature, and the supernatant was discarded. The resulting pellet was gently resuspended in MaCa buffer (0.5 M mannitol; 20 mM $CaCl_2$; 0.1% (v/v) MES pH 5.7) in a total volume yielding a protoplast density of 1 million/ml. Protoplasts were heat shocked at 45°C for 5 minutes, and allowed to cool to room temperature. 15 ml sterile Sarstedt tubes containing the DNAs for the transfection (40 µg per million cells) were prepared and 1 ml protoplast suspension was gently added to each, using cut tips. The contents in the tube were mixed and 1 ml 40% (w/v) PEG (Polyethylene-glycol 4000 dissolved in 0.1 M $Ca(NO_3)_2$, 0.4 M mannitol, pH adjusted to between 8 and 10 with KOH) was added dropwise down the wall of the tube, followed by gentle but thorough mixing before incubating at RT for 30 minutes. The protoplasts were washed by filling the tubes with W5 solution, 3 ml at a time, mixing them and adding more. They were pelleted at 100 g for 10 minutes at RT and the

supernatant was completely removed. The protoplast pellet was resuspended in 1 ml K3 and allowed to recover overnight in dark at 26°C

5.3.20.2 Radioactive labelling of protoplasts

After over night incubation of transfected protoplasts, 100 µCi/ml of ^{35}S-ProMix (Amersham) was added and incubated in the dark at 25°C for 1-3 hours. The labelling was stopped by adding unlabelled methionine and cysteine to a final concentration of 10 mM and 5 mM respectively. For pulse chase experiments, samples were taken at time intervals of 0 minute, 1 hour and 3 hours.

5.3.20.3 Protoplast homogenisation

The protoplasts were lysed on ice by adding 2 volumes of ice-cold homogenisation buffer (150 mM Tris/Cl pH 7.5, 150 mM NaCl, 1.5 mM EDTA, 1.5% TritonX-100), supplemented immediately before use with 1.5 mM PMSF, 1x anti-protease mix- Complete (Boehringer Mannheim). It was vortexed well and either frozen and stored at - 80°C or used further.

5.3.20.4 Isolation of soluble and high-speed pellet fractions from labelled protoplasts

Around 1 million protoplasts (3 ml) were put on 12% sucrose (170 µl), containing protease inhibitors (Complete tablet, Roche), in a 15 ml Sarstedt tube and mixed 50x with yellow tip and then centrifuged at 500 g for 5 min at 4°C. The supernatant was taken, out of which, 128 µl was layered over 100 µl of 17% sucrose and the rest (32 µl) was frozen as total fraction to be loaded on the gel. The supernatant layered over 17% sucrose was centrifuged at 100,000 g for 30 min at 4°C. The supernatant was taken as the soluble fraction.

5.3.20.5 Fractionation of chloroplasts isolated from labelled protoplasts

The entire procedure was conducted on ice. To the pellet of around 2 million protoplasts in a 15 ml Sarstadt tube were added 500 µl HS buffer (50 mM Hepes, 0.33 M sorbitol, pH 8). The protoplasts were disrupted by pipetting up and down 100x with a blue tip. The tube was filled till 4 ml with HS buffer and mixed 10 times with a needle attached to a 1 ml syringe. 4 ml of it was put on a 4 ml Percoll cussion (800 µl 5X HS, 1.4 ml Percoll (40%), 1.8 ml water) and centrifuged at 500 g for 8 minutes. The supernatant was removed by using a suction pump (first the interlay, containing the broken protoplasts, going down till the pellet). The pellet was washed with 8 ml HS buffer. It was then centrifuged at 1000 g for 2 minutes. The supernatant was removed by using a suction pump. The pellet was resuspended in 120 µl HS buffer and transferred to a 1.5 ml Eppendorf tube. From this, 40 µl were taken as the chloroplast fraction and frozen. Chloroplasts were treated with thermolysin to remove any externally attached protein. Chloroplasts were then pelleted at 1000 g for 1 minute and 80 µl HM buffer (10 mM

HEPES/KOH, 5 mM $MgCl_2$, pH 8) were added to it, along with 3 µl 0.5 M EDTA followed by incubation on ice for 10 minutes, followed by centrifugation at 15,500 g for 5 minutes at 4°C. The supernatant was taken as the stromal fraction. The pellet was washed with 80 µl HM buffer and centrifuged at 15,500 g for 1 minute at 4°C to remove the EDTA. The pellet was resuspended in 80 µl HMC (HM containing 2.5 mM $CaCl_2$, which quenches EDTA), 40 µl of which was taken in a fresh Eppendorf tube and frozen as the thylakoid fraction. To the rest 4 µl thermolysin (2 mg/ml dissolved in HM buffer) was added and incubated on ice for 30 minutes. 200 µl of HME (60 µl 0.5 M EDTA in 3 ml HM buffer) were added to thermolysin digestion mix and centrifuged at 15,500 g for 1 min at 4°C. The supernatant was discarded and the pellet was resuspended in 40 µl HME. This consisted of the protease protected thylakoid proteins fraction.

5.3.20.6 Immunoprecipitation of proteins from labelled protoplasts

To the samples from section 5.3.20.5 400 µl NET-gel buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.25% gelatin/ 0.05-0.1% BSA, 0.02% sodium azide) was added, mixed well and centrifuged for 4 min at 4°C at 15,500 g. The antibody was added to the supernatant and incubated on ice for 2 hours. To this was added 150 µl 10% Protein A-Sepharose and incubated for 2 hours at 4°C under gentle agitation. The beads were pelleted and washed three times with 1 ml NET-gel buffer. The proteins were eluted by adding 2x SDS-PAGE buffer and heated at 95°C for 5 minutes.

5.3.21 Isolation of nucleoids from isolated pea chloroplasts

Chloroplasts were isolated from pea leaves as mentioned above. They were pelleted in Wash Buffer I and resuspended in 5 ml of nucleoid isolation buffer (NIB: 17% w/v sucrose, 20 mM Tris/HCl, 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol, 1 mM PMSF, 1 tablet Complete (protease inhibitor cocktail from Sigma), pH 7.6) 2% Nonidet P-40 was added dropwise and allowed to stir at 4°C for 30 minutes. This suspension was then centrifuged for 10 minutes at 3000 g at 4°C. The supernatant (S1) was taken and further centrifuged at 48,000 g for 40 minutes, 4°C. The supernatant (S2) was removed and the pellet (P1) was resuspended with a syringe in 8 ml NIB. This was further centrifuged for 40 minutes at 48,000 g, 4°C. The supernatant (S3) was removed and the pellet was resuspended in 1 ml NIB, followed by another centrifugation step. The supernatant (S4) was removed. This was repeated and the pellet thus obtained, contained the nucleoid fraction and was white in colour. It was resuspended in 200 µl NIB. The different fractions thus obtained were loaded on a SDS-PAGE gel followed by western blot.

6 Results

6.1. Study of the role of phosphorylation in the transit peptide of preproteins

6.1.1 Determination of the sites of phosphorylation in the transit peptides

Chloroplast preproteins have been found to contain a phosphorylation motif which consists of the sequence (P/G)X_n(R/K)X_n(S/T)X_n(S*/T*), where n represents 0 to 3 amino acids spacer and S*/T* is the phosphate acceptor within their transit peptides. This motif was established by conducting point mutations for several proteins (Waegemann and Soll, 1996). The same experimental approach was applied in the present study to determine the sites of phosphorylation of the three *Arabidopsis thaliana* proteins, APC1, HCF136 and CAO, and pea LHCP. It involved the cloning of the point mutated constructs, thought to disrupt the site of phosphorylation in the transit peptide, into an expression vector, heterologously overexpressing them in *E. coli* cells and comparing their phosphorylations to the respective recombinant wild type proteins in a kinase enzymatic assay (section 5.2.5). The protein kinase present either in the wheat germ extract or the leaf extract of *Arabidopsis thaliana* (section 5.3.15) was used as a reactant in the phosphorylation assays. If the mutations at the putative phosphorylation sites were able to prevent the phosphorylation of preproteins, it would establish the site of phosphorylation in these proteins.

6.1.1.1 Determination of the site of phosphorylation in the transit peptide of APC1

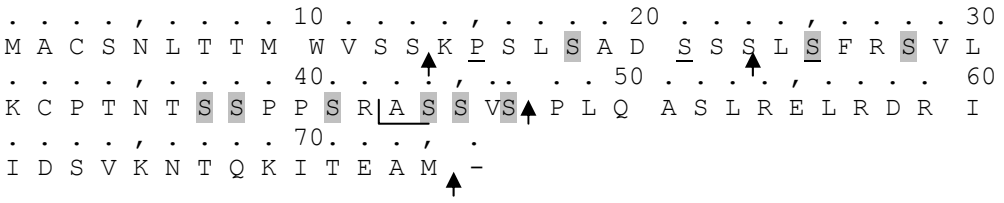


Figure 3. The predicted transit peptide of APC1 with a portion of the mature protein. (⌊___ indicates the beginning of the mature protein). Shaded amino acids represent the sites for the point mutation constructs whereas the arrows indicate the sites for the deletion constructs. Underlined residues represent the phosphorylation motif.

The *Arabidopsis thaliana* protein APC1 consists of 373 amino acids with a calculated molecular weight of 40.9 kD, of which the N-terminal transit peptide is predicted to be the initial 42 amino acids by using ChloroP 1.1 program (Emanuelsson, 1999). A partial protein sequence for APC1 is presented in Fig. 3. The full length protein was cloned into pET21a vector, overexpressed in *E. coli*, recovered in a partially purified form from inclusion bodies and subjected to *in vitro* phosphorylation assay. The phosphorylation result showed that the full length recombinant APC1 was not phosphorylated. One reason for this could be that the recombinant protein is misfolded in such a manner that the site of phosphorylation is not available to the kinase. In an attempt to obtain a more solubilised form of this protein, the N-terminal part of APC1, which included the transit peptide, was cloned in front of the mature

part of the small subunit of rubisco (mSSU) to generate the construct pAPC1#mSSU. This protein, upon subjection to a phosphorylation assay, was found to be phosphorylated although the positive charge in the motif is missing (Fig. 4A) and was used for further analysis. In the transit peptide of APC1, the serine at position 25 comes nearest to resembling the proposed phosphorylation motif, although it does not conform entirely to it. This serine was substituted by an alanine to generate the pAPC1-25SA#mSSU construct. Upon its overexpression and subjection to a phosphorylation assay, it was found to be phosphorylated. This was followed by the construction of point mutations where serines at positions 16, 18, 28, 37/38, 41, 45/46, and 47 were substituted with an alanine to give rise to pAPC1-16SA#mSSU, pAPC1-18SA#mSSU, pAPC1-28SA#mSSU, pAPC1-37/38SA#mSSU, pAPC1-41SA#mSSU, pAPC1-45/46-SA#mSSU and pAPC1-47SA#mSSU constructs, respectively. The overexpressed forms of all of these proteins were found to be phosphorylated (Fig. 4A and 4B). Further, deletions in the transit peptide of APC1 were done to find out the area of the transit peptide where phosphorylation occurs. The constructs made for this purpose were, pAPC1-Δ1-13#mSSU, pAPC1-Δ1-23#mSSU, pAPC1-Δ1-47#mSSU and pAPC1-Δ1-75#mSSU. pAPC1-Δ1-75#mSSU represented the mature part of SSU only. This construct served as a negative control to make sure that the mature part of SSU is not phosphorylated. Out of these, pAPC1-Δ1-13#mSSU and pAPC1-Δ1-23#mSSU recombinant proteins were found to be phosphorylated but not the pAPC1-Δ1-47#mSSU and pAPC1-Δ1-75#mSSU recombinant proteins. This suggested that the positions between 23 and 47 contained the site of phosphorylation (Fig. 4B and 4C). Since all the serines between 23 and 47 had already been point mutated to an alanine and were found to be phosphorylated, the serine at position 25, which represents the most likely candidate according to the reported phosphorylation motif, was mutated to a threonine (pAPC1-25ST#mSSU). After performing the standard phosphorylation reaction with the overexpressed protein of this construct, phosphoamino acid analysis was done, as described in section 5.2.5, along with the pAPC1#mSSU phosphorylated protein as a control. In case of APC1#mSSU, the phosphorylation was found to occur only at a serine, whereas for pAPC1-25ST#mSSU, the phosphorylation occured at a threonine as well as a serine (Fig. 4D). This suggests that not only the serine at position 25 is one of the sites of phosphorylation in the transit peptide of APC1, but there are one or more other serine/s as well, that is/are also implicated in the phosphorylation process.

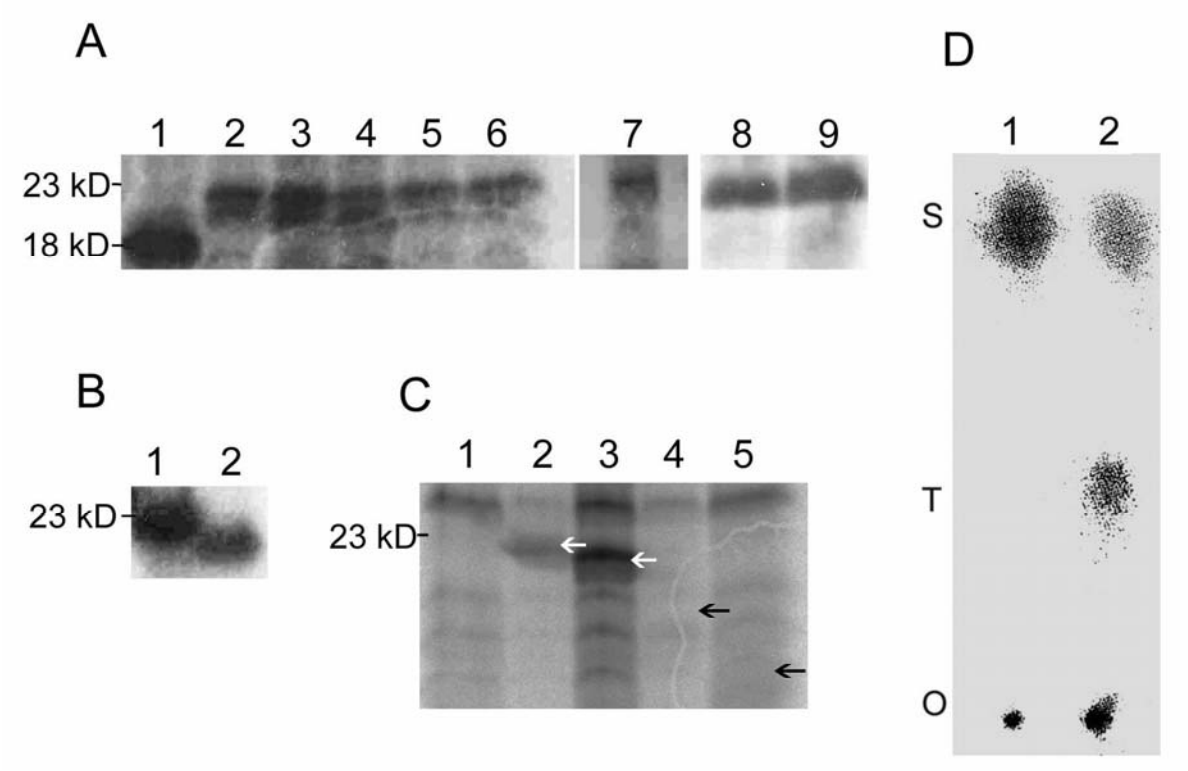


Figure 4. Determination of the site of phosphorylation in the transit peptide of APC1. A, B and C show the autoradiograms of the phosphorylation of the different mutant forms of pAPC1#mSSU. **A**. The full length of pSSU was taken as the positive control during the phosphorylation reactions (lane 1). Phosphorylation of pAPC1#mSSU (lane 2), pAPC1-16SA#mSSU (lane 3), pAPC1-25SA#mSSU (lane 4), pAPC1-28SA#mSSU (lane 5), pAPC1-41SA#mSSU (lane 6), pAPC1-18SA#mSSU (lane 7), pAPC1-44/45-SA#mSSU (lane 8), pAPC1-47SA#mSSU (lane 9). In figure **B**, lane 1 shows pAPC1#mSSU and lane 2 pAPC1-Δ1-13#mSSU. **C**. Negative control, phosphorylation reaction without any protein substrate (lane 1), pAPC1#mSSU (lane 2), pAPC1-Δ1-23#mSSU (lane 3), pAPC1-Δ1-47#mSSU (lane 4), pAPC1-Δ1-75#mSSU (lane 5). Arrows indicate the positions for the respective proteins on the SDS-PAGE gel. **D.** Phosphoamino acid analysis of the phosphorylated chloroplast precursor proteins. 1. pAPC1#mSSU and 2. pAPC1-25ST#mSSU were subjected to *in vitro* phosphorylation assay, immunoprecipitated and hydrolysed in 6 N HCl. They were then analysed by high voltage electrophoresis on silica gel thin layer chromatography plates. The positions of the phosphoamino acid standards, phosphoserine (S) and phosphothreonine (T) are indicated on left hand side. 'O' represents the origin.

6.1.1.2 Determination of the site of phosphorylation in the transit peptide of HCF136

```
. . . . , . . . . 10 . . . . , . . . . 20 . . . . , . . . . 30
M A S L Q L C D G Y  L L F K P S V S P R  F L S Q R I S H R  L
. . . . , . . . . 40 . . . . , . . . . 50 . . . . , . . . . 60
I P K A S S S  P P P  S P S P S S S S S  S L S F S R R E L L  Y
. . . . , . . . . 70 . . . . , .
Q S A A V S L S L  S S I V G P A. . .
```

Figure 5. The predicted transit peptide of HCF136 with a portion of the mature protein. ⌞ indicates the beginning of the mature protein. Shaded amino acids represent the sites for the point mutation constructs whereas the arrows indicate the sites for the deletion constructs. Underlined residues represent the phosphorylation motif.

Similar to APC1, the recombinant full length protein of HCF136 (403 amino acids in length) also did not phosphorylate in the *in vitro* phosphorylation assay. Therefore for this

protein also, the chimeric form of the protein, containing the transit peptide of HCF136 (predicted to be 60 amino acids in length, Fig. 5) and the mature part of SSU was constructed and introduced into the gateway vector pEXP2-DEST. It was overexpressed in *E. coli* cells. Upon performing the phosphorylation reaction with this protein (pHCF136#mSSU), it was found to be phosphorylated (Fig. 6B, lane2). Two deletion constructs were then generated in which the transit peptide of HCF136 was deleted at positions 18 and 37, respectively, to give rise to pHCF136-Δ1-18#mSSU and pHCF136-Δ1-37#mSSU. The overexpressed proteins obtained from these deletion constructs were subjected to the phosphorylation reaction and it was found that both of them were phosphorylated (Fig. 6A, lanes 3, 4). Thus the phosphorylation site was expected to be after position 37. The serines at positions 52 and 54 were substituted to two threonines in the same construct. Upon overexpressing and phosphorylating the protein obtained from this construct, it was found to be phosphorylated but with a much lower yield as compared to the wild type protein (Fig. 6B and 6C). Phosphoamino acid analysis showed that the phosphorylation of pHCF136-52/54-S/T#mSS occurred at a threonine. This indicated that either of the two serines at position 52 and 54 is the site of phosphorylation in the transit peptide of HCF136 protein.

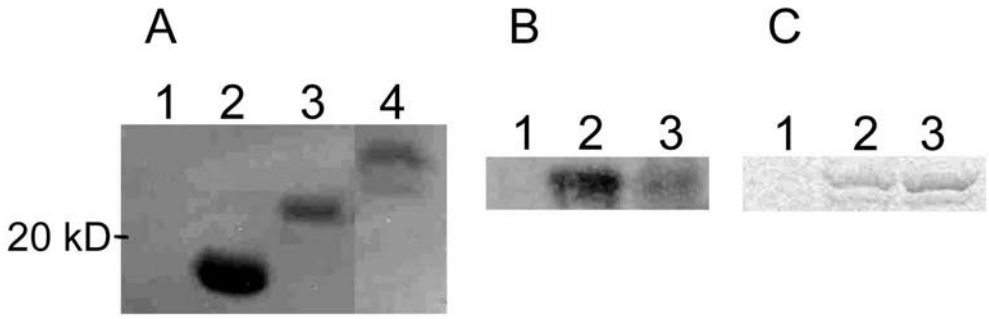


Figure 6. Determination of the site of phosphorylation in the transit peptide of HCF136. Autoradiograms showing the phophorylations for various pHCF136#mSSU constructs. **A**. Negative control for phosphorylation reaction (without a substrate) is shown in lane 1. Lane 2 shows the phosphorylated pSSU as the positive control. Lane 3 is pHCF136-Δ1-37#mSSU and lane 4 represents pHCF136-Δ1-18#mSSU. **B**. lane 1, negative control; lane2, pHCF136#mSSU; lane 3, pHCF136-52/54-ST#mSSU. **C**. Coomassie stained gel for the autoradiogram presented in 'B'.

6.1.1.3 Determination of the site of phosphorylation for CAO

```
. . . . , . . . . 10 . . . . , . . . . 20 . . . . , . . . . 30
M N A A V F S P S  A L S L P I S F S K  T R S S F L S R K K  G
. . . . , . . . . 40 . . . . ,
V K R E F R|V F A  V F G D E S
```

Figure 7. The predicted transit peptide of CAO with a portion of the mature protein. |___ indicates the beginning of the mature protein and underlined residues represent the phosphorylation motif.

CAO is a 536 amino acid protein with a transit peptide length of 36 amino acids (Fig. 7), as predicted by ChloroP 1.1. As in the case of APC1 and HCF136 proteins, CAO also

could not be phosphorylated in its full length form. Its transit peptide was also attached to the mature form of SSU to obtain pCAO#mSSU, followed by its overexpression in *E. coli*. It was then used as the substrate in a phosphorylation assay. Even this construct could not be phosphorylated. The phosphorylation reaction was performed using wheat germ or *Arabidopsis thaliana* leaf extract. Neither of the two sources of the kinase could phosphorylate pCAO#mSSU. The protein was purified over Ni-NTA and Talon columns separately to obtain a more purified form. It was also treated with 1% TritonX-100 before being used in the phosphorylation reaction. None of these different experimental approaches rendered a phosphorylated form of CAO. In order to make sure that this non-observance of phosphorylation was not due to low amount of phosphorylation, two phosphorylation reactions were carried out separately, then mixed together and subjected to denaturing immunoprecipitation as described in section 5.2.6 (Fig. 8). Even this procedure did not reveal any phosphorylation of the pCAO#mSSU construct. This led us to speculate that either the transit peptide of CAO in its overexpressed form is not susceptible to phosphorylation in the *in vitro* phosphorylation assay or this protein is not phosphorylated prior to import.

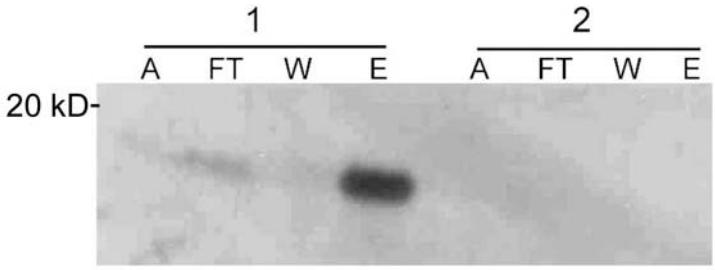


Figure 8. Denaturated immunoprecipitation of the phosphorylated proteins for pSSU (1) and pCAO#mSSU (2). 2x phosphorylation reactions for pSSU and pCAO#mSSU were performed, followed by their denaturation in 2% SDS and immunoprecipitation on Ni-NTA column with anti-SSU. 'A' is 1% of the sample applied on the column, 'FT' is the flow-through, 'W', the wash fraction and 'E' is the elution fraction.

6.1.1.4 Determination of the site of phosphorylation for LHCP

```
. . . . , . . . . 10 . . . . , . . . . 20 . . . . , . . . . 30
M A A S S S S S  M  A L S S P T L A G K  Q L K L N P S S Q E  L
                ↑
. . . . , . . . . 40. . . . , . . . . 50 . . . . , . . . . 60
G A A R F T|M R K S A ↑ T T K K V A S S  G S P W Y G P D R V  K
```

Figure 9. The predicted transit peptide of LHCP with a portion of the mature protein. ⌊ indicates the start of the mature protein. Shaded amino acids represent the sites for the point mutation constructs whereas the arrows indicate the sites for the deletion constructs.

The chloroplhyll a/b-binding protein of the light harvesting complex II (LHCP) was then taken up as a candidate to find its site of phosphorylation. This protein was earlier reported to be phosphorylated despite the absence of a phosphorylation motif in its transit peptide (Waegemann and Soll, 1996). It has 270 amino acid residues. It was cloned into the

gateway expression vector pEXP2-DEST and various deletions and point mutations were tried, as described below, to locate the phosphorylation site. The following mutant proteins were analysed: LHCP-26/27-S/A (where, in the full length LHCP protein sequence, serines at positions 26 and 27 were changed to alanines), LHCP-Δ1-8, LHCP-Δ1-41 (the deletions in the transit peptide at positions 8 and 41) and LHCP-Δ1-8-12/13-S/T (deletion of residues from 1 to 8 and substitution of serines at positions 12 and 13 to threonines) (Fig. 10A and 10B). Phospho-amino acid analysis was performed after the *in vitro* phosphorylation reactions for LHCP, LHCP-Δ1-8, LHCP-Δ1-41 and LHCP-Δ1-8-12/13-S/T to find out the residue on which the phosphorylation occurs in these proteins. The autoradiogram revealed that all the four protein constructs are phosphorylated at serine as well as threonine residues (Fig. 10C). An endogenous phosphorylation site with threonine as the phosphate acceptor is present very close to the N-terminus of mature LHCP. This site has been reported to be involved in the regulation of light harvesting in the thylakoids *in vivo* (Bennett, 1991). Thus this phosphorylation site might also be recognised in the *in vitro* phosphorylation experiments. Therefore, the transit peptide of LHCP was fused with the mature part of mSSU (pLHCP#mSSU) in order to overcome the phosphorylation of threonine in the mature sequence. pLHCP-26/27-S/A#mSSU, pLHCP-Δ1-8#mSSU and pLHCP-Δ1-8-12/13-S/T#mSSU clones were then constructed followed by their use in an *in vitro* phosphorylation assay. The transit peptide of LHCP was found to be phosphorylated in each case (Fig. 10D). Upon performing phosphoamino acid analysis of pLHCP#mSSU and pLHCP-Δ1-8#mSSU, both these proteins were also found to be phosphorylated at both serine as well as threonine residues (Fig. 10E). The transit peptide of LHCP has two threonines and nine serines out of which five serines are at positions four to eight, which were not present in the deletion mutant pLHCP-Δ1-8#mSSU. Therefore, pLHCP is able to phosphorylate at either or all of the amino acid positions 12, 13, 15, 26, 27 and 36 in the *in vitro* phosphorylation assay. The exact or tentative site of phosphorylation could not be determined in this case.

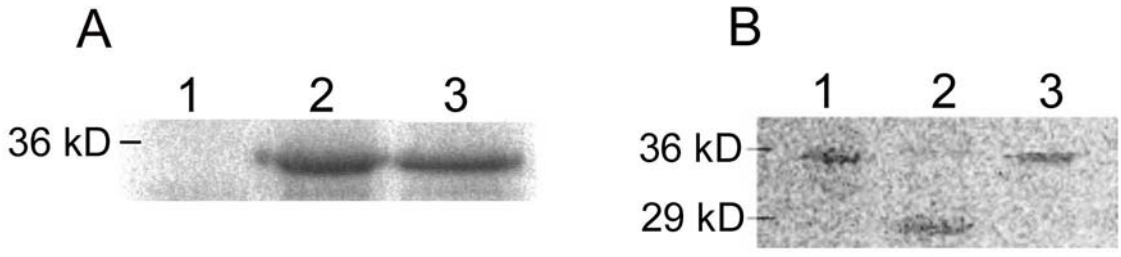
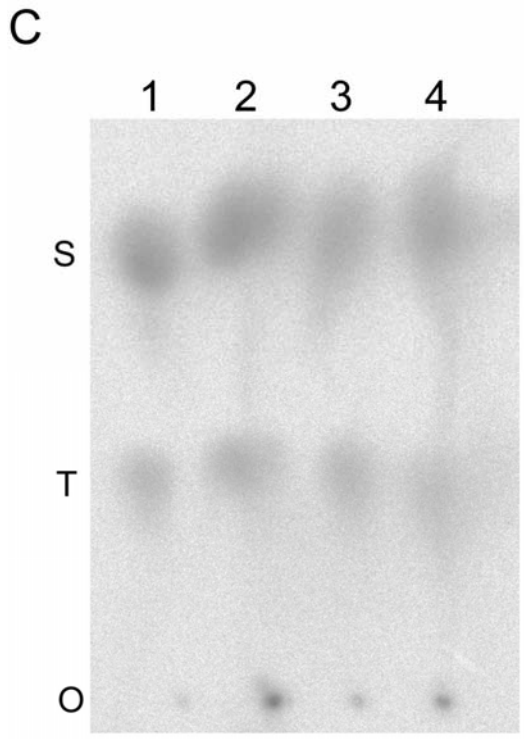
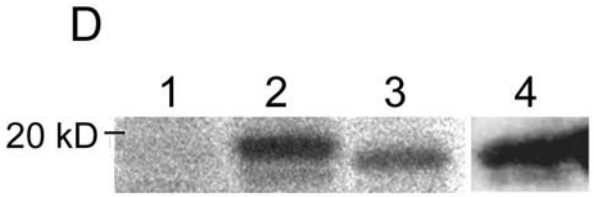


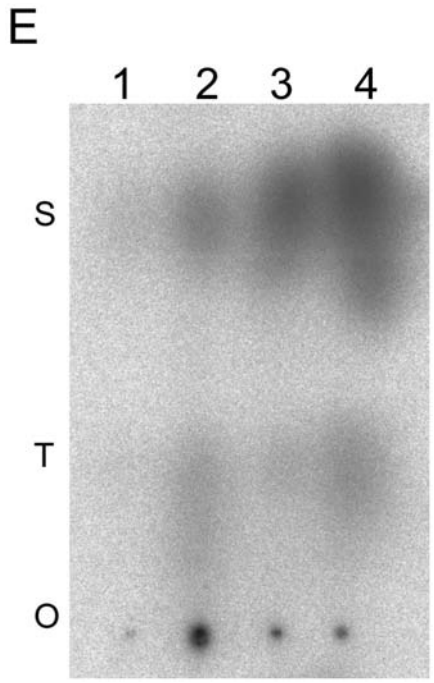
Figure 10. Determination of the site of phosphorylation in the transit peptide of LHCP. Autoradiograms for the LHCP constructs, as indicated. **A**. Lane 1, negative control; lane 2, LHCP; lane 3, LHCP-26/27-S/A. **B**. Lane 1, LHCP-Δ1-8; lane 2, LHCP- Δ1-41; lane 3, LHCP-Δ1-8-12/13-S/T.



C. Phosphoamino acid analysis for LHCP (lane 1), LHCP-Δ1-8 (lane 2), LHCP- Δ1-47 (lane 3) and LHCP-Δ1-8-12/13-S/A (lane 4). 'O' is the site of origin for the migration of the amino acids. 'S' and 'T' indicate the positions for phosphoserine and phosphothreonine, respectively.



D. Autoradiograms for the negative control (lane 1), pLHCP#mSSU (lane 2), pLHCP-Δ1-8#mSSU (lane 3), pLHCP-26/27-S/A#mSSU (lane 4).



E. Autoradiogram of the phosphoamino acid analysis experiment for the negative control (lane 1), LHCP (lane 2), pLHCP#mSSU (lane 3) and pLHCP-Δ1-8#mSSU (lane 4).

6.1.2 Attempts to study the effect of phosphorylation on the import of preproteins

The experimental approach applied to study the significance of phosphorylation in the transit peptide upon the import of preproteins consisted of the establishment of a homologous system using *Arabidopsis thaliana* as the experimental plant system. It was

planned to study the transport of preproteins from the cytoplasm to the chloroplast surface *in vivo* and study the effect of the mutated forms of these proteins (i.e. lacking the site of phosphorylation), upon the transport process, on a real-time basis. It is known that the impairment of the site of phosphorylation does not impede targeting of the preprotein to the chloroplast *in vitro* (Waegemann and Soll, 1996). The phosphorylated precursor forms a complex with a 14-3-3 protein and an Hsp70 isoform which has been shown to increase the rates of their import (May and Soll, 2000). Therefore, it was hypothesized that the mutant forms, lacking the sites of phosphorylation, might be retarded and their accumulation in the cytosol could be observed. On the other hand, the transfer of wild type proteins was expected to be comparatively much quicker. A number of techniques, as described below, were applied to approach this problem.

6.1.2.1 Microinjection of mRNA/DNA into isolated protoplasts.

6.1.2.1.1 Construction of GFP-fusion constructs.

For the purpose of the localisation of the proteins in a cell, C-terminal GFP/RFP fluorescent fusion probes were constructed. To begin with, pSSU#mLHCP-GFP (where pSSU represents the presequence of SSU; mLHCP, the mature part of LHCP) and pSSU31/34-SA#mLHCP-RFP (where pSSU31/34-SA is the presequence of SSU with the serines at positions 31 and 34 substituted by alanines; mLHCP is the mature part of LHCP) constructs were made by introducing the inserts into pOL-GFP/ pOL-RFP vector. This construct was chosen because the mutation at the phosphorylation site of pSSU has been previously shown to completely inhibit the phosphorylation of the protein (Waegemann and Soll, 1996) and mLHCP is a hydrophobic protein and would slow down the transport of the chimeric protein into the chloroplast, as compared to the rapid transport of the full length pSSU. These constructs were transformed into isolated protoplasts of *Arabidopsis thaliana* or tobacco to check for their expressions. The expressed proteins were observed through fluorescent microscope. Both the constructs showed similar localisations within chloroplasts of transfected protoplasts (Fig. 11) indicating that the lack of phosphorylation site in the transit peptide of preproteins does not impair its targeting to chloroplasts, *in vivo* as well.

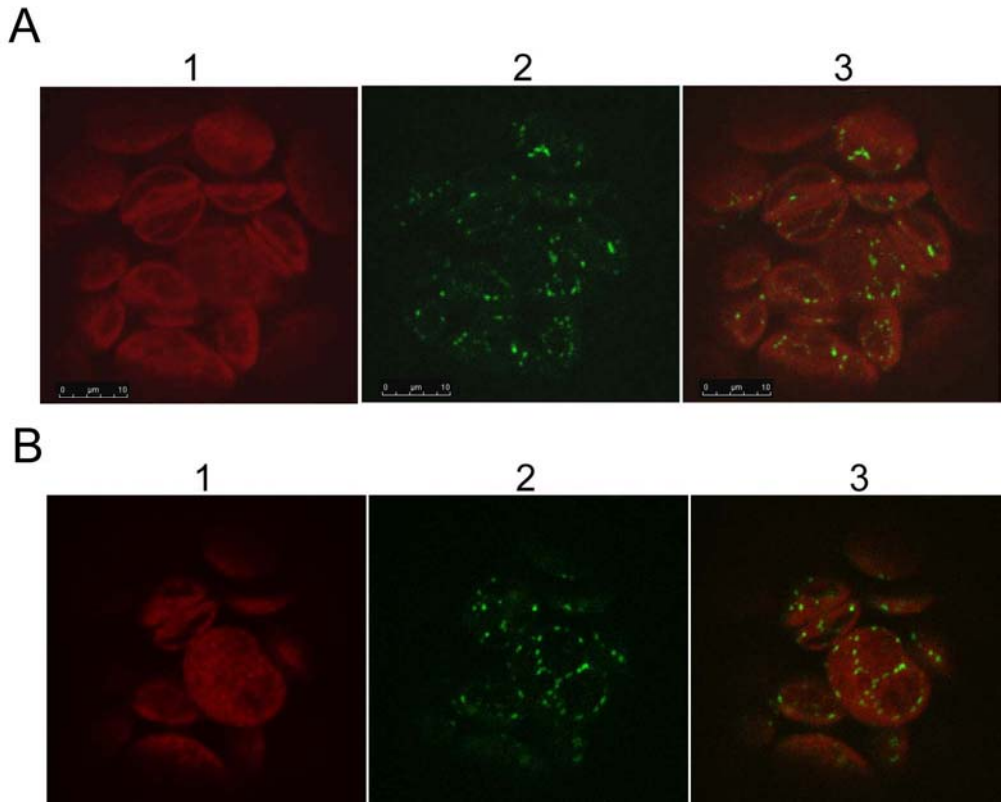


Figure 11. Transient expression of pSSU#mLHCP-GFP (A) and pSSU-31/34-S/A#LHCP-RFP (B) in isolated protoplasts. Protoplasts were transformed with the pOL plasmid containing the above mentioned inserts using the PEG transformation method (section 5.3.11). Lanes 1 show the individual protoplasts with chlorophyll autofluorescence (Red), lanes 2 show them with GFP/RFP fluorescence and 3 is the overlap of 1 and 2.

Further, the aim was to inject mRNA/DNAs into isolated protoplasts to observe their expression on a real-time basis. For the purpose of obtaining mRNA of the GFP/RFP fused constructs, the following approach was applied. Since the pOL-GFP and pOL-RFP vectors contain a plant expression promoter (CaMV 35S) which cannot be used for the *in vitro* transcription, a T7 promoter was cloned in front of the inserts to obtain the mRNA for the chimerical proteins, pSSU#mLHCP-GFP and pSSU-31/34-S/A#LHCP-RFP. The constructs within the pOL vector were as depicted in Fig. 12.

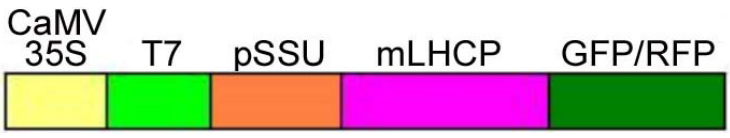


Figure 12. The insert cloned with T7 promotor into the pOL-GFP/ pOL-RFP vector for the purpose of *in vitro* transcription to obtain mRNA for microinjection. CaMV 35S is the plant expression promoter present in pOL vector, T7 promoter was cloned into the vector before the inserts consisting of either pSSU#mLHCP-GFP or pSSU-31/34-S/A#mLHCP-RFP.

6.1.2.1.2 The microinjection technique and the prerequisites for it.

A number of prerequisites are necessary and need to be optimised for microinjecting mRNA/DNA into isolated protoplasts. Since this was the first attempt at the application of this technique in our laboratory, it will be described in some detail here.

1. Plant material, isolation and maintenance of protoplasts

Protoplasts from *Arabidopsis thaliana*, tobacco and sometimes pea leaves were used for the following experiments. After extensive study, it was found that the tobacco protoplasts from the leaves of 3 week old plants are better suited for the purpose of microinjection than that of *Arabidopsis* and pea protoplasts because of their bigger size and higher stability.

A number of media were tried to find a suitable medium for the isolation and storage of protoplasts in suspension. Tobacco protoplasts were isolated as described in section 5.3.9. After the gradient step, the protoplasts were resuspended in either F-PCN or transformation buffer and incubated in dark. The protoplasts in F-PCN survived longer (two to three days) than those in transformation buffer (one day). Upon putting a drop of this protoplast suspension on the 'injection plate' (section 4.12), the medium was found to start evaporating within a few minutes, leading to a change in the osmolarity of the medium and the rapid death of the protoplasts. Overlaying the protoplast suspension drop with paraphenol oil was tried to avoid the evaporation of the medium. This was not suitable because when the injection needle was introduced from above the oil drop, the oil attached to it, leading to it being useless for the microinjection of the protoplasts. On the other hand, when the oil drop was added after the injection needle was in place in the 'injection plate', despite it being introduced very gently, it moved the position of the needle and further hampered its mobility. The other approach used to overcome this problem of changing osmolarity was to find a suitable osmoticum, in the presence of which the longevity of protoplasts is increased comparatively. Sucrose, glucose and mannitol were added to MS medium. Mannitol was found to give better results, although the viability of the protoplasts was still less in terms of the time span required for microinjection. The molarity of mannitol was varied to see its effect upon the protoplasts. 0.4 M mannitol in MS medium was found to be optimum for the longevity of protoplasts.

2. Microinjection technique

Injection needle: Femtotips and Femtotips II, the two kinds of injection capillaries provided by Eppendorf, were tried for the injection purpose. Femtotips II were found to be better than Femtotips, due to their thinner wall and being more finely pulled. In a protoplast suspension, the needle gets blocked very easily. The dilution of the solution to be injected is also important as its higher viscosity can lead to the blockage of the needle. In order to view the

opening of the injection capillary, a fluorescence dye was added to the solution to be effect in the protoplast suspension. If the concentration of the dye in the injection solution was not enough, it was not possible to observe any difference between the injected and the non-injected protoplasts (Fig. 15). The dye injected into the protoplast also differentiates the injected protoplasts from the non-injected ones. FITC (fluorescein isothiocyanate) and Lucifer yellow were tried for this purpose, but FITC was later discarded because it was found to stain the plasma membrane without being injected into the protoplasts (Fig. 16).

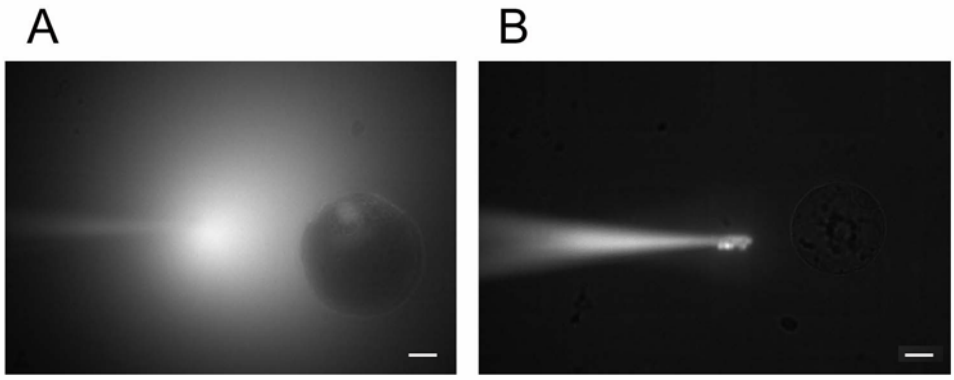


Figure 13. Visibility of the opening of the injection capillary using dyes. Fluorescent dyes were added to the injection solution in order to differentiate a blocked needle from an unblocked one. **A**. An open injection capillary in position for microinjection of a protoplast. **B**. A blocked capillary. The fluorescence of the dye is limited to within the injection capillary, indicating that it is blocked at its tip. The tip also shows the presence of debris attached to it. Scale bars represent 10 µm.

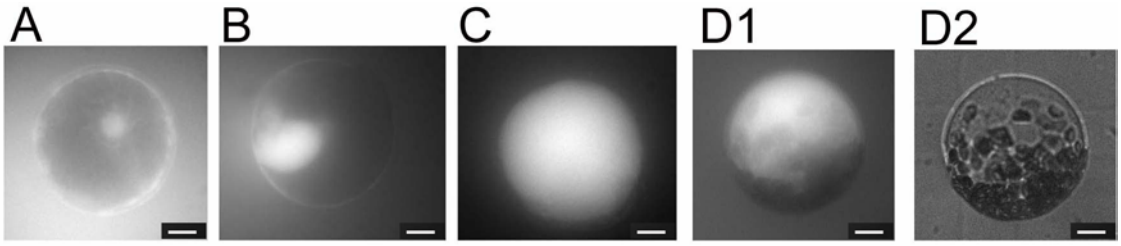
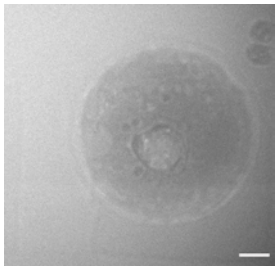


Figure 14. Microinjected protoplasts of tobacco with mRNA/DNA and lucifer yellow. The success of microinjection is indicated by the presence of the dye, within them. D1 and D2 are the same protoplast, D1 is shown under fluorescent light, whereas D2 is shown under transmission light. Scale bars represent 10 µm.

Figure 15. An injected protoplast, which could not be differentiated from non-injected ones because of the low amount of dye in the injection solution. The scale bar represents 10 µm.



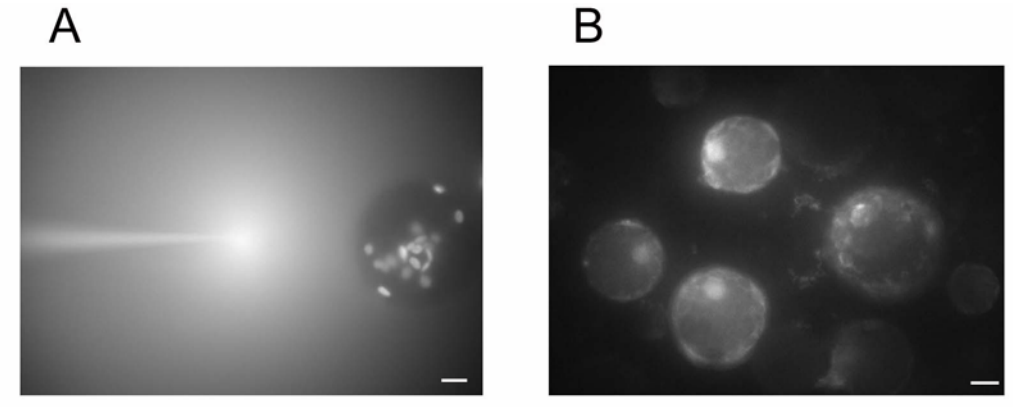


Figure 16. Microinjection of protoplasts with injection solution containing FITC. **A**. Both FITC and chlorophyll autofluorescence could be detected by the FITC filter used. **B**. Isolated protoplasts which were not injected with FITC show the effects of microinjection. Scale bars represent 10 µm.

Holding capillary: Using a holding capillary, although the protoplast could be held in position most of the time, the required pressure from the suction pump could not be maintained for long. The pressure is critical for the proper holding and viability of the protoplast. Slightest movement in the medium around the protoplast can change the required pressure, leading to either the release of the protoplast or its suction into the capillary. In cases this could be properly controlled, when the injection capillary was moved towards the protoplast, the same problems arose, as the pressure required to hold the protoplast in position changed. Another major problem was the visibility of the holding and the injection capillaries at the same time. At 10x magnification, the holding capillary was in proper view, but a higher magnification is required for the injection process to be conducted properly. At 40x magnification, although the injection needle is in proper focus, the holding capillary along with the protoplast to be injected lost their focus. It was not possible to view both the capillaries, along with the protoplast to be injected in sharp focus simultaneously. Various angles and lengths of the holding capillary and the injection capillary were tried to overcome this problem, but without success. In addition, the holding capillary lost its focus due to the mechanical movement caused when the view was changed from transmission light to fluorescence filter (Fig. 17).

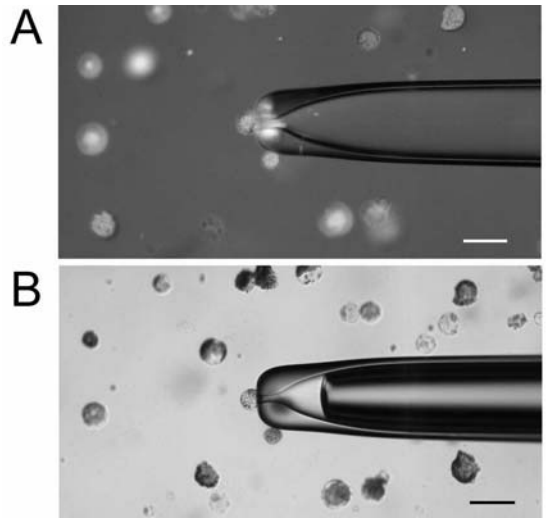


Figure 17. Visibility of the holding capillary under different light conditions but same focus. **A**. Under fluorescence light for the observation of chlorophyll autofluorescence. **B**, under transmission light. Scale bars represent 100 µm.

In view of these difficulties, the attachment of the protoplasts to the 'injection plate' containing the protoplast suspension was considered as a possible better option over the use of the holding capillary.

Attachment of protoplasts

Three different techniques have been described in the literature for the attachment of protoplasts. Two of them involve the embedding of protoplasts in alginic acid or agarose, whereas the third involves their attachment to glass using poly-L-lysine.

1. Thin-alginate-layer technique – The embedding of protoplasts in alginate was done as described in section 5.3.16. This technique proved to be unsuitable for microinjections because of a number of reasons. The injection needle either broke or was blocked as soon as it was inserted into the alginate layer. A certain amount of manoeuverability of the needle is required to adjust the position of the needle to be in focus with the protoplast to be injected. This kind of movement was not possible inside the alginate layer. Although the protoplasts in alginate remained healthy for a longer period of time, they were in more than one layers and their visibility over the inverted microscope was therefore compromised.
2. Embedding protoplasts in ultra-low melting point agarose (section 5.3.17). The problem of blocked needles was also observed in this case but most of the time the microinjection of the protoplasts was successfully performed. But the injected protoplasts did not survive long after the injection.

   In both cases, the protoplasts are confined to a limited space surrounded by the embedding medium. During injection, when the solution is injected into it, the walls of the protoplast expand a little to adjust the added volume. This natural expansion of the protoplast due to microinjection is hindered while being embedded in alginate layer or agarose, leading to their almost immediate death.
3. Poly-L-lysine coating of glass-slips for the attachment of protoplasts - Many different dilutions of the 0.1% stock solution of poly-L-lysine were tried to find the optimal percentage required for the attachment of protoplasts. 1:400 dilution of the stock solution in distilled water was found to be optimal for the attachment and longevity of the protoplasts. In addition, it was found that the attachment worked much better only when K3 medium was used for the isolation and incubation of the protoplasts and sea water (section 5.3.18) was used for the washing steps in the procedure for the attachment (Fig. 18). One problem that was encountered during the microinjection process was that the protoplasts in the vicinity of the holding and the injection capillary detached from their positions and attached to the capillaries due to electrostatic forces, making them lose their flexibility of movement (Fig. 19). Another problem was the attachment of chloroplasts or other organelles within the protoplasts

to the injection needle, when it pierced through the protoplast plasma membrane. This led to the rupture of the protoplast being injected (Fig. 20A), in addition to the injection needle being useless for further injections, as the attached organelle did not detach from the needle on its way out of the protoplast (Fig. 20B).

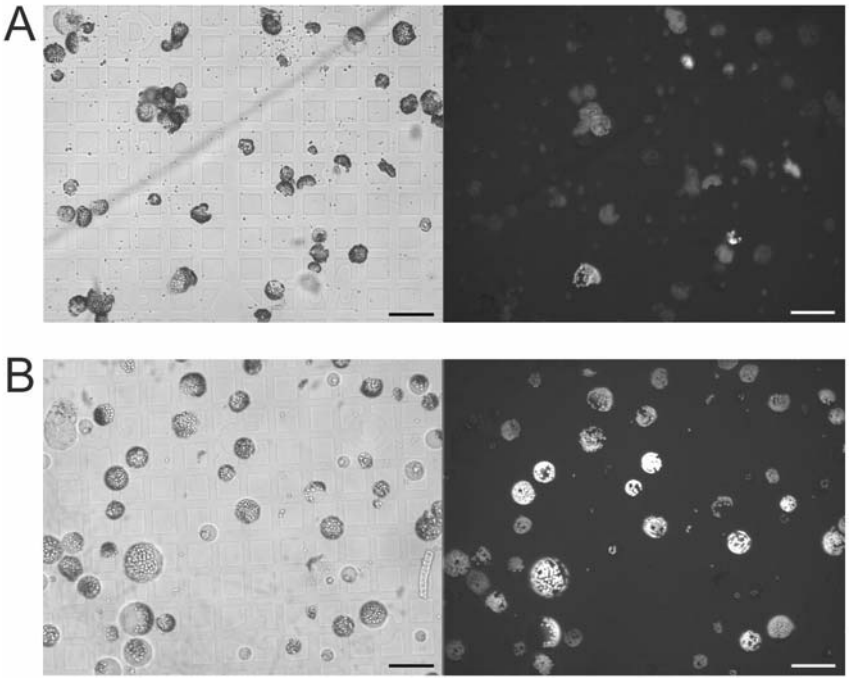


Figure 18. Attachment of protoplasts on glass-slips using poly-L-lysine. Protoplasts were isolated in either F-PIN (A) or K3 medium (B) and attached to glass slips using 1:400 dilution of poly-L-lysine. They were incubated in the respective media over night and observed the next day under the microscope. The pictures on the left side show the attached protoplasts under transmission light, whereas the ones on the right side show the chlorophyll autofluorescence. Scale bars represent 100 µm.

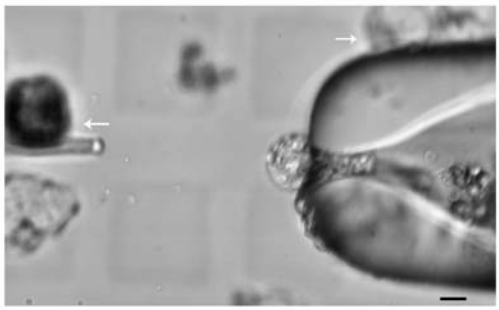


Figure 19. The effect of electrostatic forces leading to the attachment of floating protoplasts and debris to the injection and the holding capillary. The scale bar represents 10 µm.

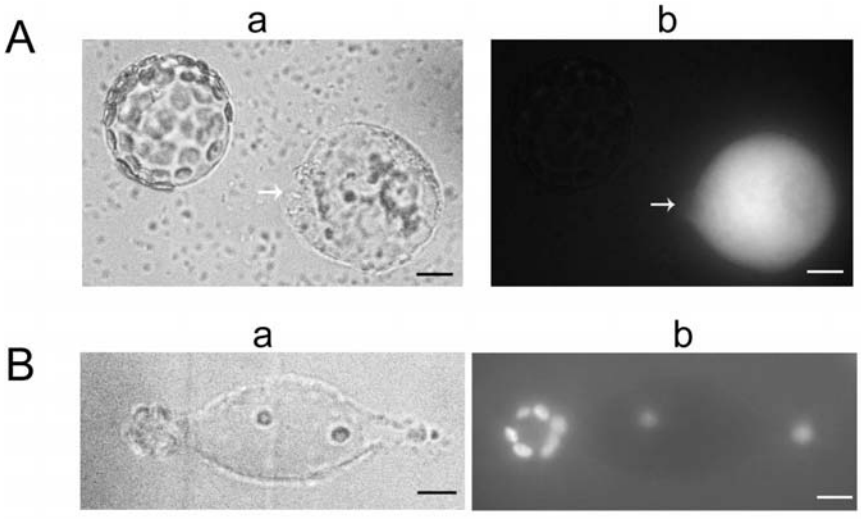


Figure 20. Disruption of protoplasts as a result of microinjection. The protoplasts are shown in, (a) transmission light and (b) fluorescence light. **A**, A part of the plasma membrane is disrupted at the site of injection. **B**, While injecting, an organelle within the protoplast attached to the injection capillary. It remained attached to it when the capillary was pulled out of the protoplast, leading to the stretching of the protoplast. Scale bars represent 15 µm.

The attachment of protoplasts with poly-L-lysine was found to be most suitable for microinjection of attached protoplasts. Using this approach, the protoplasts could be successfully microinjected. One to seven protoplasts could be injected with the same needle, and 20-30 protoplasts could be injected per hour. But the expression of the constructs used for the injection of the protoplasts, either as DNA or as mRNA, was not observed, even after 12-18 hours after injection. Most of the time, the injected protoplasts either died during the incubation period or they did not get transformed. The entire procedure seemed to be too stressful for the protoplasts.

6.1.2.1.3 *In vivo* labelling of protoplasts and analysis of expressed proteins

Due to the microinjection technique being unsuccessful for observing the difference in the expression levels and the transport of the wild type and the mutant forms of the proteins into chloroplasts, *in vivo* labelling technique was tried. For this purpose, pSSU#mLHCP, pSSU-31/34-SA#mLHCP; OE23 (the full length 23 kD subunit of the oxygen-evolving complex), OE23-22SA (OE23 with a point mutation from serine to an alanine at its site of phosphorylation, as reported by Waegemann and Soll, 1996) and pOE23-GFP, pOE23-22SA-GFP constructs were used (section 5.1.3). The GFP constructs were chimeras of the transit peptides of OE23 or OE23-22SA and the green fluorescent protein (GFP). The four OE23 constructs were a kind gift from Prof. Colin Robinson, University of Warwick, UK.

This procedure consisted of a number of steps starting with the isolation of protoplasts from 3-4 week old tobacco plants followed by their transformation with the above mentioned constructs using the PEG transformation technique as described in section 5.3.20.1. The transformed protoplasts were then allowed to recover for 16 hours by incubating them at 25ºC in the dark. They were then radio-labelled with ^{35}S containing methionine and cysteine for 1 to 3 hours and immunoprecipitated using the antibody against the respective proteins. Anti-LHCP antibody was used for the constructs pSSU#mLHCP and pSSU-31/34-SA#mLHCP; anti-OE23 antibody for OE23 and OE23-22SA and anti-GFP antibody was used for pOE23-GFP and pOE23-22SA-GFP constructs. It has been shown that pOE23-GFP construct is correctly targeted to chloroplasts in transfected tobacco protoplasts (Di Cola and Robinson, 2005).

The labelling experiments for pSSU#mLHCP and pSSU-31/34-SA#mLHCP did not give satisfactory results probably due to the antibody not being able to precipitate its target protein (Fig. 21). In case of both pairs of constructs for OE23, it was found that the expression levels of the wild type as well as the mutant form were similar after pulse-chase experiment (section 5.3.20) (Fig. 22).

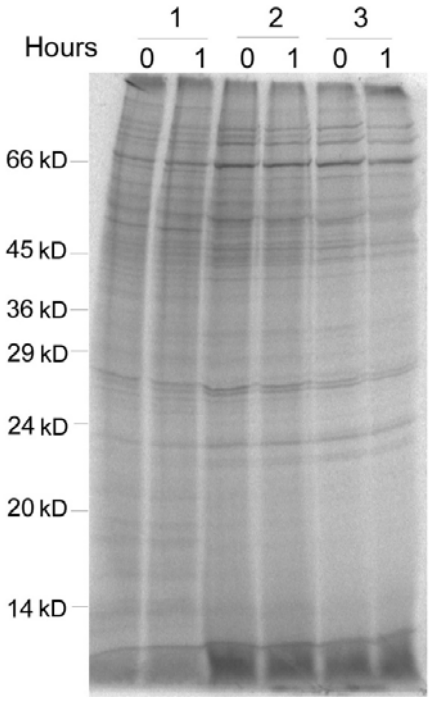


Figure 21. The autoradiogram showing the pulse chase experiment for negative control (1), in which the protoplasts were mock transformed with water; pSSU#mLHCP (2) and pSSU-31/34-S/A#mLHCP (3). Isolated protoplasts were transformed with these constructs, incubated overnight and labelled with ^{35}S-Met and ^{35}S-Cys isotope for 1 h and the labelled proteins were then chased with a mixture of cold Met and Cys for the times indicated above each lane. The protoplasts were then lysed and subjected to immunoprecipitation using antibodies against LHCP.

One significant observation from these experiments was that in case of the *in vivo* labelled protein OE23-22SA, an additional protein band at around 5-8 kD was observed (Fig. 22, lanes in 3). This band did not occur in case of the wild type OE23 (Fig. 22, lanes in 2). Such an extra band was not observed in the case of pOE23-GFP and pOE23-22SA-GFP proteins (Fig. 22, lanes in 6). The intermediate and mature forms of the proteins in the autoradiogram show a slight difference for the full length (lanes 1-3) and the GFP constructs (lanes 4-6), because the GFP protein is higher in molecular weight than the OE23 protein. Upon fractionation of the OE23 and OE23-22SA transfected protoplasts into soluble and pellet fractions, most of this lower molecular weight protein was found to be in the pellet obtained after high speed centrifugation (Fig. 23, lanes 1-3). Further, when chloroplasts were isolated from the transfected protoplasts and fractionated into the stromal and the thylakoid fraction, this lower molecular weight protein band was found to be in the total chloroplast fraction and the thylakoid fraction (Fig. 23, lanes 4-6). It was found to decrease after thermolysin treatment of the thylakoid fraction. Since this band appears only when the full length OE23 protein contains a point mutation at its phosphorylation site, it indicates that this residue may play an important role in the import behaviour of the full length protein. As the point mutation is in the stromal-directing transit peptide of OE23, theoretically it is cleaved off in the stroma and should not reach the thylakoids. Also, upon fractionation of chloroplasts, this band was not found in the stroma fraction at all. From these observations, it seems probable that the band in question is somehow associated with the envelopes. Its observation with thylakoids could be due to the fact that in this fractionation procedure, the envelopes were not separated from the thylakoids. Unfortunately this could not be confirmed by performing proteolysis with thermolysin of the total chloroplasts fractions, due to the lack of the antibody required for immunoprecipitation. Several attempts were made to perform this

experiment with the available antibody against pea OE23 derived from chicken, but although the proteins could be labelled, the immunoprecipitation experiments did not work.

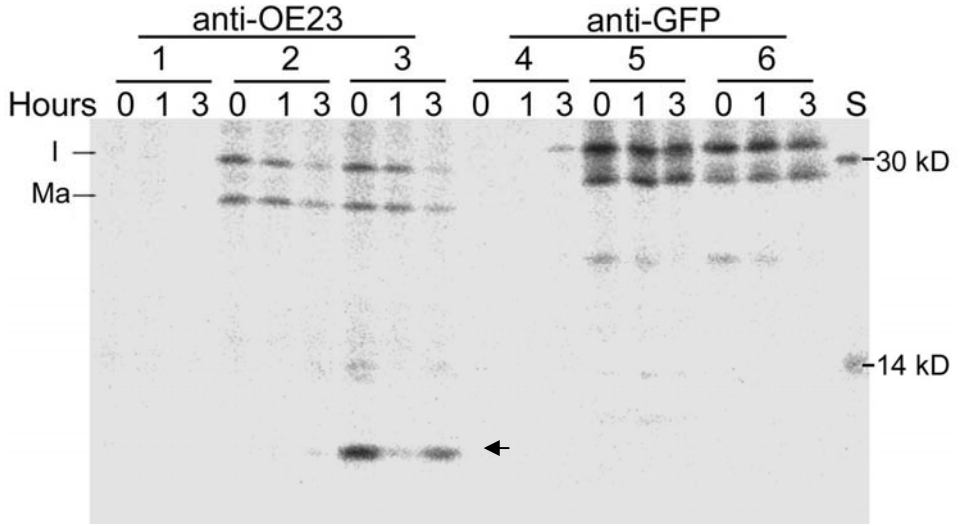


Figure 22. Pulse chase experiments for OE23 (2), OE23-22SA (3) and pOE23-GFP (5), pOE23-22SA-GFP (6). Protoplasts were isolated from the leaves of 3-4 week old tobacco plants and transformed with the above mentioned constructs. After overnight incubation in the dark, the protoplasts were pulse labelled with ^{35}S-Met and ^{35}S-Cys isotopes for 1 h and then chased with a mixture of cold Met and cold Cys for the times indicated above each lane. The protoplasts were then lysed and subjected to immunoprecipitation using antibodies against OE23 or GFP, as indicated. 1 and 4 are the negative controls, consisting of protoplasts mock transformed with water. 'I' is the intermediate and 'Ma' is the mature form of the protein. The arrow indicates the extra band observed for OE23-22SA protein.

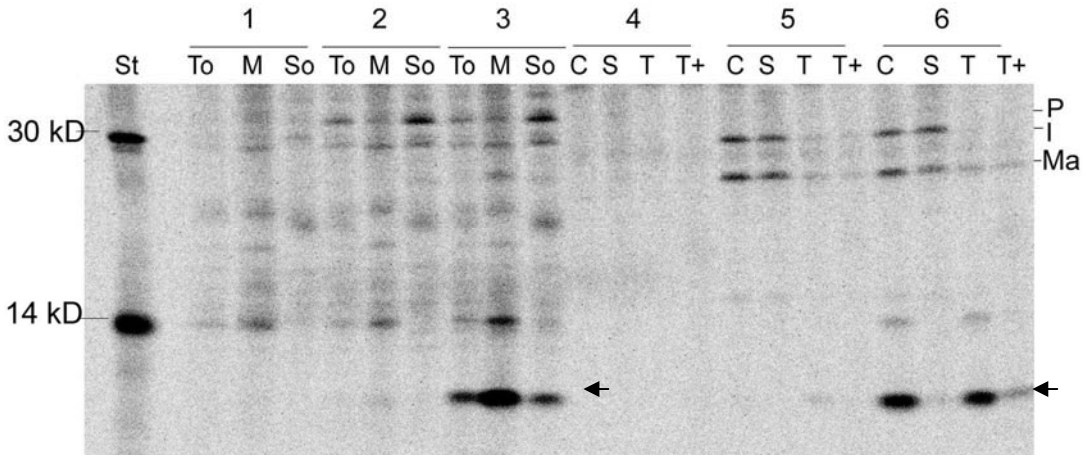


Figure 23. Protoplasts expressing OE23 and OE23-22SA were radio-labelled with ^{35}S-Met and ^{35}S-Cys isotope followed by fractionation. 1,2,3 are fractionations into pellet (M) and soluble (So) fractions for negative control, OE23 and OE23-22SA, respectively. 'To' is the total protoplasts. 4,5,6 are the fractionations for negative control, OE23 and OE23-22SA into chloroplasts (C) stroma (S) and thylakoids (T). T+ represents the thermolysin digested thylakoid fraction. 'P' is preprotein, 'I' is intermediate and 'Ma' is mature protein. 'St' is the standard marker and the arrows indicate the extra band observed for OE23-22SA.

The GFP-fused constructs when transfected into isolated protoplasts did not reveal any difference in the localisations of the wild type and the mutated OE23 within the protoplasts (Fig. 25). The same was the case for protoplasts transfected with pSSU-GFP and pSSU-

31/34SA-GFP (Fig. 24) despite their observance under the microscope after varied periods of time. Therefore, GFP fused proteins were not considered to be appropriate to study the effect of phosphorylation of transit peptides upon the import of preproteins into chloroplasts. On the other hand, the usage of the full length proteins (OE23 and OE23-22SA) in the *in vivo* studies, by applying the radio-labelling technique, could provide some insights into this process. Further work needs to be done to be able to confirm the importance of this phosphorylation process before import of preproteins into chloroplasts.

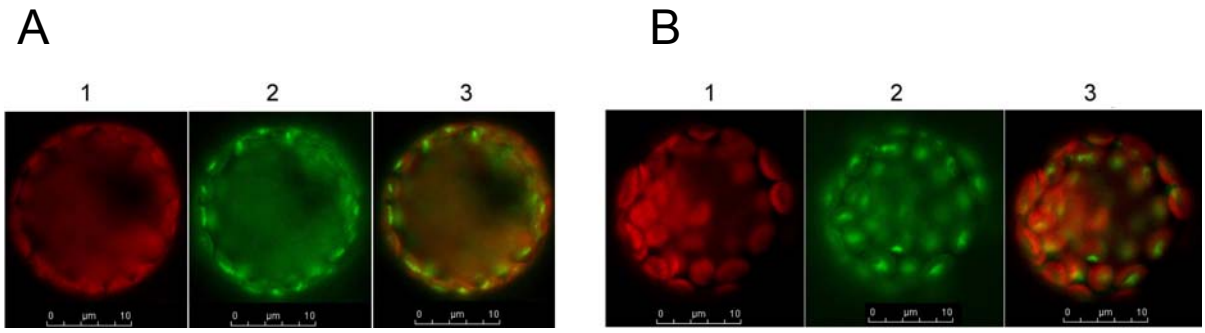


Figure 24. Isolated protoplasts transformed with pSSU-GFP (A) and pSSU31/34-SA-GFP (B). Lane 1 shows the chlorophyll autofluorescence; lane 2 the GFP expressing protein and lane 3, the overlay of the images in 1 and 2.

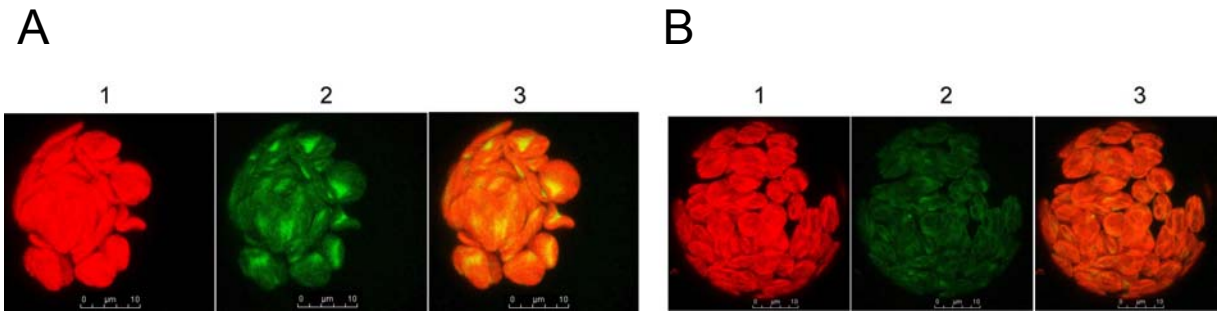


Figure 25. Isolated protoplasts transformed with pOE23-GFP (A) and pOE23-22SA-GFP (B). Lane 1 shows the chlorophyll autofluorescence, lane 2 the GFP expressing protein and lane 3, the overlay of pictures 1 and 2.

6.2 The characterisation of the two forms of psNDPK2 and the study of the localisation of atNDPK2

6.2.1 Detailed study of the two proteins encoded by the psNDPK2 gene.

6.2.1.1 Primary structure of psNDPK2

The psNDPK2 is a 25 kD protein which comprises of 231 amino acids. It was amplified from pea cDNA using gene specific primers and inserted into the pET21a vector. It was then sequenced (Fig. 26). The sequence result revealed that it contained an alanine at position 77 instead of a threonine, as given in the database (Swissprot: P47923). It was then *in vitro* transcribed, and translated in the wheat germ or reticulocyte lysate system. Upon import of the radio-labelled translation product into isolated chloroplasts, two bands were obtained at positions 18.5 kD and 17.4 kD respectively, the higher band being less prominent than the lower one (Fig. 27, lane 2). This result was in concurrence with the earlier published results of Luebeck and Soll, 1995. Further, to investigate the discrepancy of our sequencing results from that of the published data, a point mutation was performed at position 77 in which the alanine at this position was changed to a threonine to obtain the psNDPK2-77AT construct. Upon import of the *in vitro* translated product of psNDPK2-77AT into isolated chloroplasts, the proportion of the two bands was seen to be reversed (Fig. 27, lane 6). Further, from the examination of the alignment of the NDPK2s from different plant species, as presented in Fig. 46, it was observed that all the other NDPK2 isoforms have an alanine at this position. From these results it was concluded that an alanine is present at position 77 of psNDPK2 and the published sequence of pea NDPK2 (Swissprot: P47923) is incorrect with regard to this position.

```
    M  E  A  M  A  V  F  S  G  S  N  L  F  A  T  S  S  L  L  L
    ATGGAAGCCATGGCCGTGTTTTCCGGAAGCAATCTCTTTGCTACATCCTCTCTCTTACTC
  1 ---------+---------+---------+---------+---------+---------+
    TACCTTCGGTACCGGCACAAAAGGCCTTCGTTAGAGAAACGATGTAGGAGAGAGAATGAG

    T  T  N  S  K  T  R  Y  S  Q  L  R  T  T  Q  N  L  S  A  F
    ACCACCAACTCCAAAACCAGATACTCTCAATTACGCACCACCCAAAACCTATCTGCATTT
 61 ---------+---------+---------+---------+---------+---------+
    TGGTGGTTGAGGTTTTGGTCTATGAGAGTTAATGCGTGGTGGGTTTTGGATAGACGTAAA

   S  S  K  S  H  L  F  S  P  S  S  T  S  S  S  Y  P  K  T  F
    TCTTCTAAATCCCATCTCTTCTCGCCTTCTTCTACTTCTTCTTCCTATCCTAAAACCTTC
121 ---------+---------+---------+---------+---------+---------+
    AGAAGATTTAGGGTAGAGAAGAGCGGAAGAAGATGAAGAAGAAGGATAGGATTTTGGAAG
           63                                  74        77
    R  T  R  S  S  T  E  S  G  I  F  L  P  R  L  I  A  S  L  E
    CGCACCAGAAGCAGCACCGAATCCGGCATCTTCCTTCCCCGCCTAATCGCTTCTCTCGAA
181 ---------+---------+---------+---------+---------+---------+
    GCGTGGTCTTCGTCGTGGCTTAGGCCGTAGAAGGAAGGGGCGGATTAGTGAAGAGAGCTT

    Q  V  D  Q  A  Y  I  M  V  K  P  D  G  V  Q  R  G  L  V  G
    CAAGTTGACCAAGCTTACATTATGGTCAAACCTGATGGTGTGCAACGTGGCCTTGTGGGT
241 ---------+---------+---------+---------+---------+---------+
    GTTCAACTGGTTCGAATGTAATACCAGTTTGGACTACCACACGTTGCACCGGAACACCCA

    E  I  I  S  R  F  E  K  K  G  F  K  L  T  G  L  K  L  F  Q
    GAAATTATTTCTAGGTTTGAGAAAAAGGGGTTTAAGTTAACTGGCTTGAAGCTCTTTCAG
301 ---------+---------+---------+---------+---------+---------+
    CTTTAATAAAGATCCAAACTCTTTTTCCCCAAATTCAATTGACCGAACTTCGAGAAAGTC

    C  S  K  E  L  A  E  E  H  Y  K  H  L  N  Q  K  S  F  F  P
    TGCTCTAAAGAATTAGCTGAGGAGCATTACAAACACCTAAACCAAAAGTCTTTCTTCCCT
```

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361 ---------+---------+---------+---------+---------+---------+
    ACGAGATTTCTTAATCGACTCCTCGTAATGTTTGTGGATTTGGTTTTCAGAAAGAAGGGA

    K  L  I  E  Y  I  T  S  G  P  V  V  S  M  A  W  E  G  V  G
    AAGCTGATTGAATATATTACTTCAGGTCCCGTTGTGTCTATGGCATGGGAGGGTGTTGGA
421 ---------+---------+---------+---------+---------+---------+
    TTCGACTAACTTATATAATGAAGTCCAGGGCAACACAGATACCGTACCCTCCCACAACCT

    V  V  P  S  A  R  K  L  I  G  A  T  D  P  L  Q  A  E  P  G
    GTAGTTCCATCGGCACGCAAGCTTATAGGGGCTACGGATCCTCTTCAAGCTGAACCAGGC
481 ---------+---------+---------+---------+---------+---------+
    CATCAAGGTAGCCGTGCGTTCGAATATCCCCGATGCCTAGGAGAAGTTCGACTTGGTCCG

    T  I  R  G  D  F  A  V  Q  T  G  R  N  I  I  H  G  S  D  S
    ACGATAAGAGGAGACTTTGCTGTTCAAACAGGAAGGAATATTATTCATGGCAGTGACAGC
541 ---------+---------+---------+---------+---------+---------+
    TGCTATTCTCCTCTGAAACGACAAGTTTGTCCTTCCTTATAATAAGTACCGTCACTGTCG

    P  E  N  G  E  R  E  I  A  L  W  F  K  E  G  E  L  C  E  W
    CCTGAAAATGGCGAGCGTGAAATAGCTCTATGGTTCAAGGAAGGCGAATTATGCGAATGG
601 ---------+---------+---------+---------+---------+---------+
    GGACTTTTACCGCTCGCACTTTATCGAGATACCAAGTTCCTTCCGCTTAATACGCTTACC

    T  P  V  Q  E  P  W  L  R  E  *
    ACTCCCGTCCAAGAACCATGGCTAAGAGAATAA
661 ---------+---------+---------+---
    TGAGGGCAGGTTCTTGGTACCGATTCTCTTATT
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Figure 26. The cDNA and deduced amino acid sequence of psNDPK2 (using AnnHyb v.4.934). Amino acids 1-64 represent the organellar targeting sequence. The high-molecular-weight form of psNDPK2 starts at position 65 whereas the low-molecular-weight form starts at position 78 (Luebeck and Soll, 1995).

6.2.1.2 Import of psNDPK2 into isolated chloroplasts

In the previous study conducted by Luebeck and Soll (1995), psNDPK2 was isolated from chloroplasts and the isolated fraction was found to contain two active forms of the protein. They obtained the N-terminal protein sequence of the two polypeptides and determined that the 18.5 kD mature form starts at position 65 and the 17.4 kD form begins at position 78. In order to characterise these two different forms of the psNDPK2, the processing of the two forms was impaired separately by applying the following approach. It has been reported that a positively charged amino acid, usually an arginine, is present within 10 amino acids before the site of cleavage, at the C-terminal region of the transit peptides of chloroplast-bound proteins (Gravel and von Heijne, 1990). Archer and Keegstra (1993), showed that the substitution of this positively charged arginine residue to a negatively charged residue in the transit peptide region of pSSU resulted in impaired processing of the protein. On the basis of these reports, mutations were constructed for the psNDPK2. The arginines at positions 63 and 74, which occur just before the start of the higher (at position 65) and the lower (position 78) forms of the protein (Fig. 26), were substituted by an aspartate to give rise to the two constructs- psNDPK2-63RD and psNDPK2-74RD. To evaluate the effects of these alterations on the import characteristics of the point mutated proteins, import reactions were performed. It was observed that in each case only one form of the protein appeared, whereas the processing of the other form was effectively impaired (Fig. 27). In case of the psNDPK2-74RD construct, the imported product was a little higher in molecular weight than that observed in the wild type protein. A possible reason for this could

be that the processing site in this case is not identical to the wild type form. These constructs were further used to study the two forms in detail.

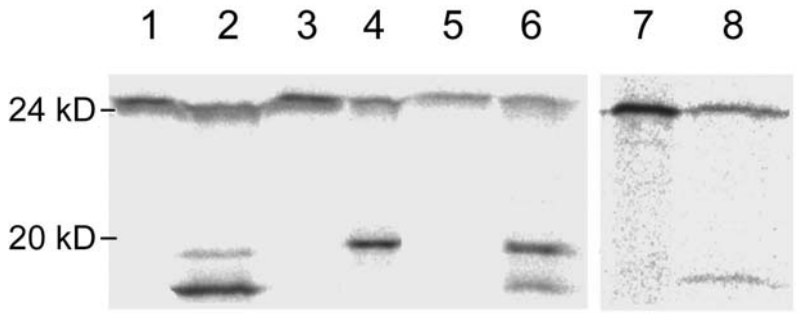

Figure 27. Import of psNDPK2, psNDPK2-74RD, psNDPK2-77AT and psNDPK2-63RD into chloroplasts. Intact pea chloroplasts (equivalent to 20µg chlorophyll) were incubated with ^{35}S-labelled *in vitro* translated protein for 15 min in the presence of 3 mM ATP at 25°C. Chloroplasts were isolated as described previously (Waegemann and Soll, 1991). Lanes 1, 3 ,5, and 7 show one-tenth of the translation product used for import reactions, lanes 2, 4, 6 and 8 are the import reactions of psNDPK2, psNDPK2-74RD, psNDPK2-77AT and psNDPK2-63RD, respectively.

6.2.1.3 Import characteristics of the two forms of psNDPK2

In order to further investigate the kinetics of the import of the two forms of the enzyme, the import experiment was performed in a series of time scale (Fig. 28). For both the forms, the imported product was seen to increase gradually with time in a similar proportion.

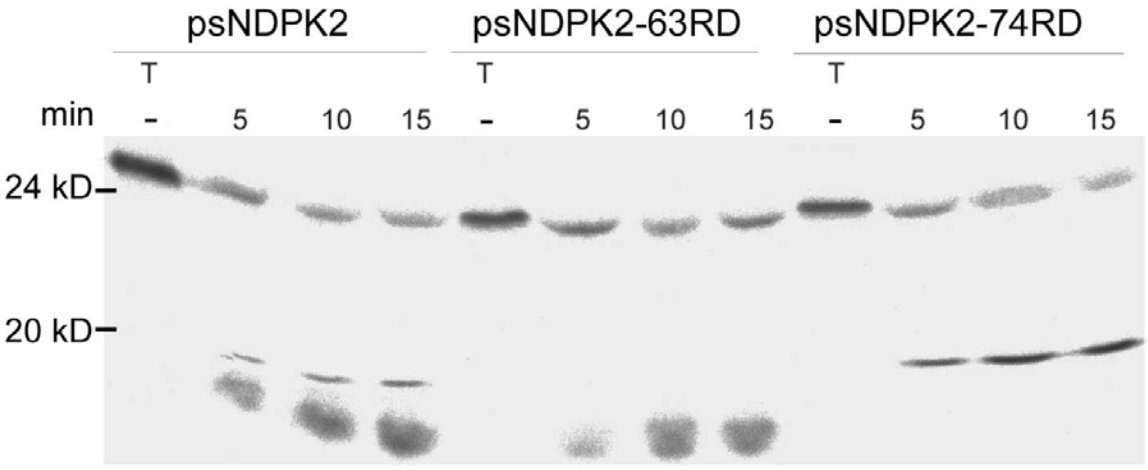

Figure 28. Time-course import for psNDPK2, psNDPK2-63RD and psNDPK2-74RD. The *in vitro* translated products were subjected to import reactions for varied periods of time. 'T' is one-tenth of the translation product used in each import reaction.

Further, to find out the energy requirement during import for the two forms, the proteins from the three constructs under study were allowed to be imported into chloroplasts in the presence of different concentrations of ATP (Fig. 29). The endogenous ATP from chloroplasts was depleted by incubating them in the dark for half an hour before the import reaction was performed in the dark. The ATP present in the translation product was depleted

by using ATP depletion columns (section 4.11). As low as 10 µM ATP was found to be enough for the import of psNDPK2, psNDPK2-63RD and psNDPK2-74RD into chloroplasts. The rate of import increased gradually with the increase of the ATP concentration in the import reaction, but the ratios were proportional for both the upper and the lower forms of the protein.

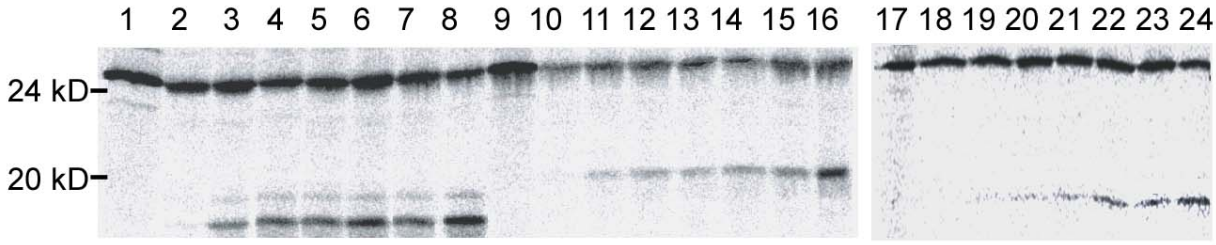

Figure 29. ATP dependency for import of psNDPK2, psNDPK2-63RD and psNDPK2-74RD into chloroplasts. ATP was depleted from chloroplasts by incubating them in the dark for half an hour before performing the import reaction. ATP present in the translation product was depleted by using ATP depletion columns (see section 4.11). psNDPK2 (lanes 1-8) psNDPK2-74RD (lanes 9-16) and psNDPK2-63RD (lanes 17-24) were allowed to be imported into isolated chloroplasts in the presence of varying amounts ATP i.e. no ATP (lanes 2, 10, 18), 10 µM (lanes 3, 11, 19), 20 µM (lanes 4, 12, 20), 50 µM (lanes 5, 13, 21), 100 µM (lanes 6, 14, 22), 500 µM (lanes 7, 15, 23), 3 mM (lanes 8, 16, 24) ATP. Lanes 1, 9 and 17 represent one-tenth of the translation product used in each reaction.

Next, the age of the plant was varied for the isolation of chloroplasts to be able to study its effect upon the import behaviour of the two forms of psNDPK2. Chloroplasts from the leaves of 5-days old and 10-days old pea plants were isolated simultaneously. In case of the 5-days old plants, only the top-most leaves, which were still in an unfolded state, were used for the isolation. Import of the *in vitro* translated proteins psNDPK2, psNDPK2-63RD and psNDPK2-74RD was carried out into the two types of chloroplasts, isolated from younger and older plants, in parallel (Fig. 30). The autoradiogram of the import reactions showed that both the upper and the lower forms of the protein are imported with similar efficiency. The only difference observed between the import into younger and older chloroplasts is that in all cases, the import efficiency of younger chloroplasts is higher as compared to the ones from older chloroplasts. The reason for this could be due to the difference in the chlorophyll contents of the two types of chloroplasts. The size of the younger chloroplasts is smaller than the older ones due to which their chlorophyll content may be lower than their older and bigger counterparts. And since the amount of chloroplasts added to the two types of import reactions was calculated by their chlorophyll contents, the number of chloroplasts in the import reaction using younger chloroplasts could be higher than that for the older chloroplasts, which may have led to the higher import efficiency in the former case.

The above experiments revealed that the two forms of psNDPK2 showed no difference in their import behaviours.

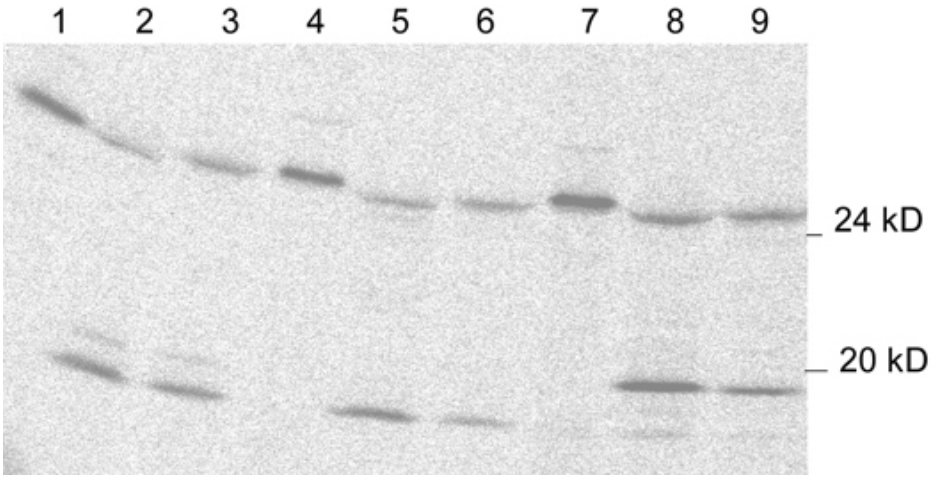

Figure 30. Effect of varying the age of chloroplasts upon the import process of psNDPK2 (lanes 1-3), psNDPK2-63RD (lanes 4-6) and psNDPK2-74RD (lanes 7-9). Chloroplasts for import reaction were isolated from the leaves of 5-days and 10-days old pea plants. Lanes 1,4 and 7 represent one-tenth of the translation product used for the import reaction; lanes 2, 5 and 8 are the import reactions using chloroplasts from 5-days old plants, whereas lanes 3, 6 and 9 represent the imports with chloroplasts from 10-days old plants.

6.2.1.4 Suborganellar localisation of the two forms of psNDPK2

To further characterise the two forms of psNDPK2, the investigation into their subcellular localisations was undertaken by conducting fractionation experiments. After performing import reactions for psNDPK2 and psNDPK2-63RD, chloroplasts were lysed and centrifuged at high speed to obtain the soluble (supernatant) and the membrane (pellet) fractions of the chloroplast, as described in section 5.3.5. Upon loading the samples on SDS-PAGE gel, followed by autoradiography, most of the proteins were seen to be in the soluble fraction (Fig. 31). Since some amount of the proteins were also observed in the membrane fractions, import and fractionation as above was followed by Na_2CO_3 extraction for psNDPK2 and psNDPK2-74RD in order to study their attachment to membranes. After Na_2CO_3 extraction, all the proteins were dissociated from the membranes (Fig. 32). These experiments revealed that both forms of psNDPK2 are in the soluble fraction of chloroplasts.

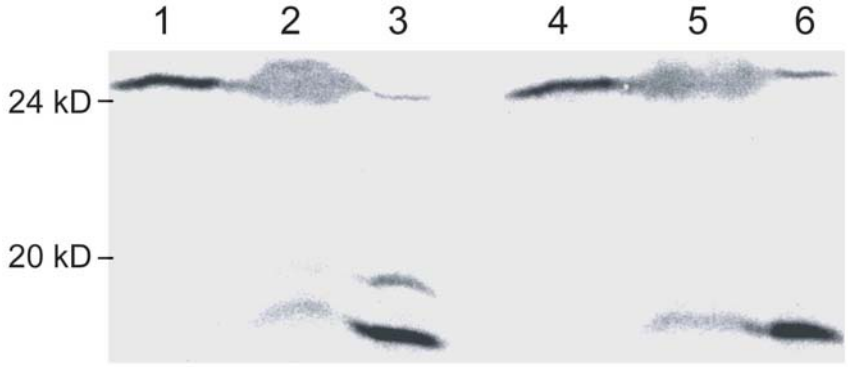

Figure 31. Fractionation of psNDPK2 (lanes 1-3) and psNDPK-3RD (lanes 4-6) into membrane and soluble fractions. *In vitro* import of psNDPK2 and psNDPK2-63RD was performed into isolated chloroplasts. Chloroplasts were then lysed with 10 mM Hepes/KOH pH 7.6 on ice followed by centrifugation at 174,000 g for 15 min, 4°C. Lanes 1 and 4 show one-tenth of the translation product used in the import reaction, lanes 2 and 5 are the pellet fractions and lanes 3 and 6 are the soluble fractions.

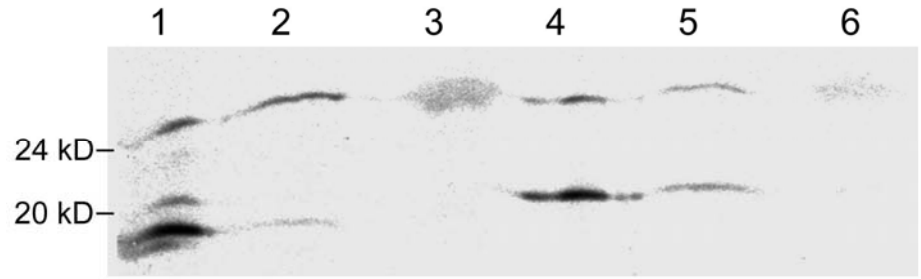

Figure 32. Fractionation of psNDPK2 (lanes 1-3) and psNDPK2-74RD (lanes 4-6) into membrane and soluble fractions, followed by Na_2CO_3 extraction of the membrane fraction. Import reaction was performed for psNDPK2 and psNDPK2-74RD into isolated chloroplasts and chloroplasts were pelleted. The pellet was resuspended in 10mM Hepes/KOH, pH 7.6 on ice, followed by centrifugation at 174,000 g for 15 min, 4°C. Lanes 1 and 4 represent the supernatant. Na_2CO_3 extraction was performed with the pellet and it was centrifuged at 256,000 g for 10 min. Lanes 2 and 5 are the supernatants after this centrifugation and lanes 3 and 6 are the pellets.

Since fractionation results revealed that both forms of psNDPK2 are soluble proteins, they could be present in the intermembrane space between the chloroplast envelopes, the stroma or the thylakoid lumen. The next step was to find out in which of these compartments are the two proteins located.

In order to check for the presence of the two forms of psNDPK2 in the intermembrane space of chloroplasts, psNDPK2, psNDPK2-63RD and psNDPK2-74RD were imported into chloroplasts and the re-isolated chloroplasts were digested with trypsin as described in section 5.3.4. In parallel, trypsin digestion was carried out for isolated chloroplasts followed by western blot analysis to check the proteolysis efficiency (Fig. 33A). The blots obtained from this experiment revealed that Toc34 (outer envelope protein) is digested after 2 minutes and Tic110, Tic62 and Tic22 (inner envelope proteins, out of which Tic110 has an intermembrane domain, Tic62 is at the stromal side of the envelope and Tic22 is largely in the intermembrane space) decrease gradually with increasing time of proteolysis. In addition to the translation products of the above mentioned constructs, the translation products for either LHCP (a thylakoid protein) or FNR (a stromal protein) were added to the import reaction (Fig. 33B and 33C). These two controls helped to make sure that the stromal and thylakoid proteins were not digested in the reaction, whereas the proteins of the outer envelope of chloroplasts are completely and those of the inner envelope are partially digested. The proteins of the intermembrane space should also be digested in these experiments and a decrease in their amounts should be observed with increasing time of trypsin digestion. After loading the samples on SDS-PAGE and autoradiography, no decrease in the amounts of the imported products was observed for the two forms of psNDPK2, LHCP and FNR after digesting chloroplasts with trypsin. These results lead us to propose that neither of the two forms of psNDPK2 are localised in the intermembrane space of the chloroplast.

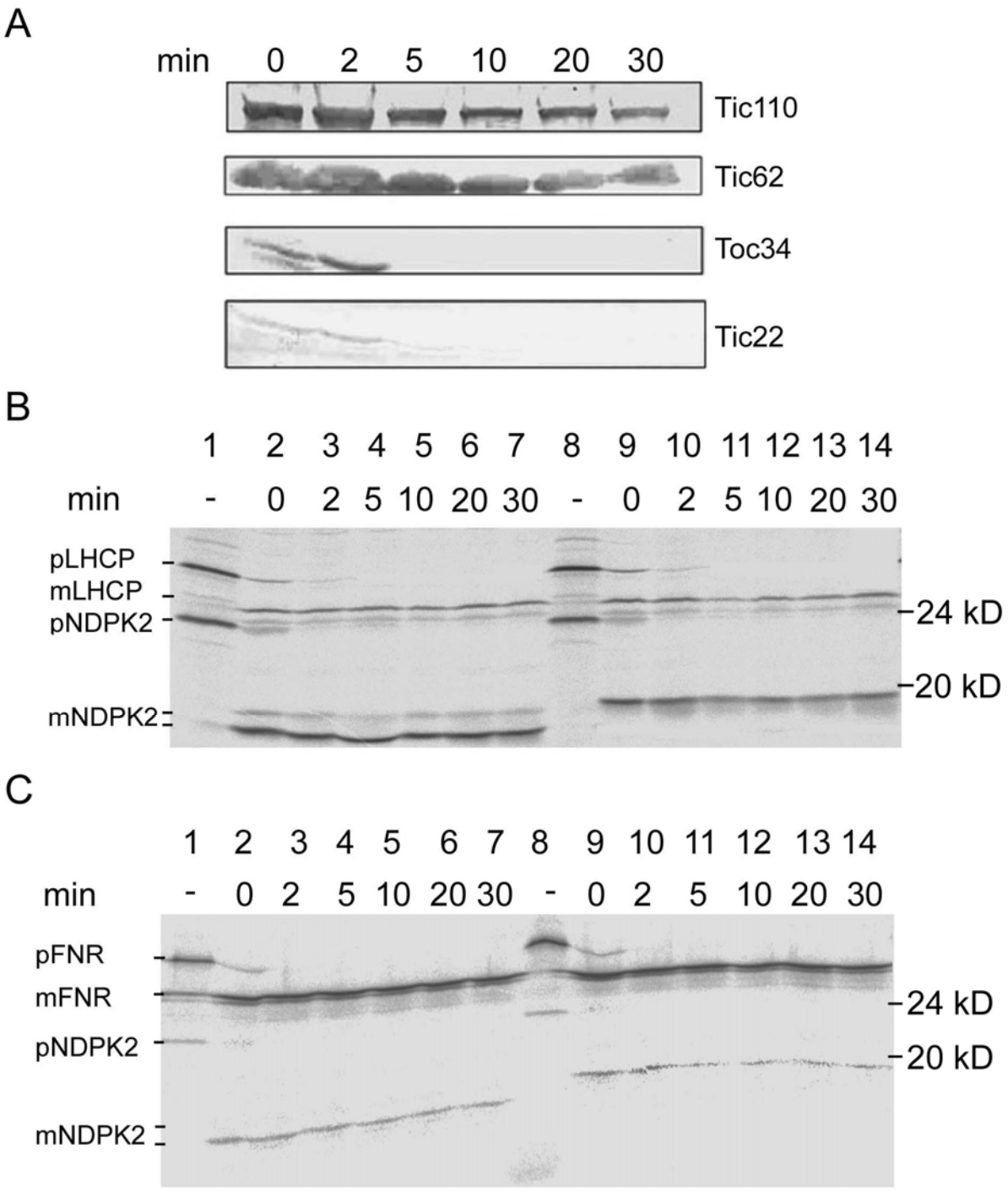

Figure 33. Trypsin digestion of intact chloroplasts after import. **A**. 20 µg chlorophyll-containing chloroplasts were digested with trypsin (1µg/µg chlorophyll) for the time periods indicated above the figure followed by western blot analysis using antibodies against Tic110, Tic62, Toc34 and Tic22. **B and C**. Chloroplast import reactions were performed with psNDPK2, psNDPK2-63RD and psNDPK2-74RD. The washed chloroplasts were then digested with trypsin (1 µg /µg of chlorophyll) for variable periods of time to digest the outer membrane of the chloroplast and the proteins of the intermembrane space. Proteolysis was stopped by adding 10x trypsin inhibitor. **B**. Along with psNDPK2 (lanes 1-7) and psNDPK2-74RD (lanes 8-14), *in vitro* translated LHCP protein was also added to each reaction mixture as a control to make sure that the stromal proteins are not proteolysed. Lanes 1 and 8 show one-tenth of the translation product used in each import reaction. The trypsin digestion was allowed to take place for the time indicated above the respective lanes. **C**. Similar to Fig. B, except that the translation products of psNDPK2-63RD and psNDPK2-74RD were used for the reactions along with FNR as the control.

Since both the forms of psNDPK2 are possibly not localised in the intermembrane space, as could be inferred from the results of the above experiment, they could either be in the stroma or the thylakoid lumen. In order to look into this possibility, import reactions were carried out for psNDPK2 and psNDPK2-74RD, followed by the fractionation of chloroplasts into stroma, envelopes and thylakoids according to the procedure described in section 5.3.6 (Fig. 34). No protein was observed in the envelope fraction whereas sizable amounts of the proteins were seen to be present in stroma and thylakoid fractions. Both forms of psNDPK2 were in similar proportions in the two fractions. Further, to investigate the attachment of the proteins to the thylakoid membrane, import reactions were performed for all the 3 constructs (psNDPK2, psNDPK2-63RD and psNDPK2-74RD), followed by the fractionation of chloroplasts as above. This was followed by the Na_2CO_3 extraction of the thylakoid fraction. The intactness of the thylakoids was confirmed by performing western blot analysis using antibody against OE23 with the different fractions (Fig. 35A). The autoradiograms from this experiment revealed that all of the protein, earlier found to be in the thylakoid fraction, dissociates from the thylakoids upon Na_2CO_3 treatment (Fig. 35B). This is true for both the forms of the protein and indicates that they are possibly not present in the thylakoid lumen. This experiment also revealed that most of the proteins are found to be extractable from the thylakoid fraction and only a minor amount is in the stroma. These results indicate that psNDPK2 protein is extrinsically associated with the stroma-facing region of the thylakoids.

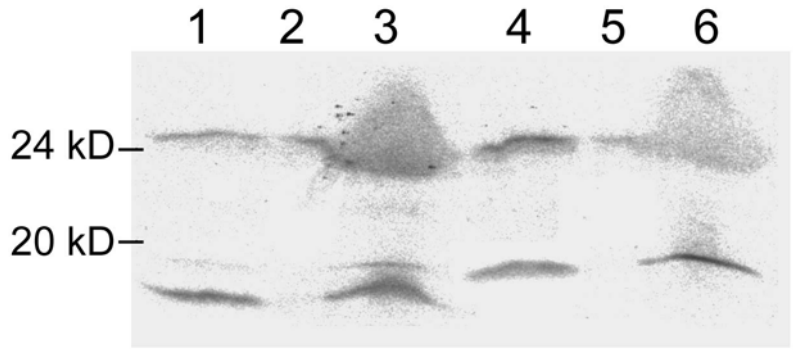

Figure 34. Suborganellar-localisation of psNDPK2 (lanes 1-3) and psNDPK2-74RD (lanes 4-6) in chloroplasts. *In vitro* translated psNDPK2 and psNDPK2-74RD were imported into isolated chloroplasts. These chloroplasts were then fractionated into stroma, envelopes and the thylakoid fractions. Lanes 1 and 4 represent the stroma; lanes 2 and 5, the envelopes and lanes 3 and 6 show the thylakoid fractions.

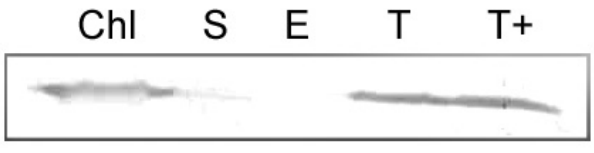

Figure 35A. Immunoblot analysis of the different fractions of chloroplast using antibody against OE23. 'Chl' are chloroplasts, 'S'- stroma, 'E'- envelopes, 'T'-thylakoids, 'T+'- thylakoids after proteolysis with thermolysin.

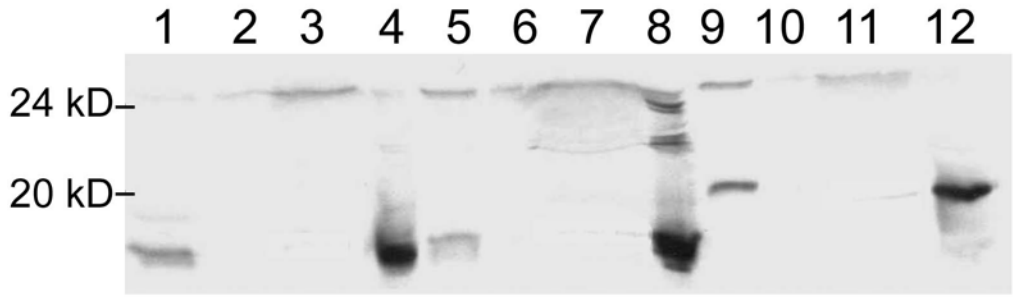

Figure 35B. Import, fractionation and Na_2CO_3 extraction of psNDPK2 (lanes 1-4), psNDPK2-63RD (lanes 5-8) and psNDPK2-74RD (lanes 9-12). 8x import reactions were performed for the three proteins followed by the fractionation of chloroplasts into stroma (lanes 1, 5, 9), envelopes (lanes 2, 6, 10) and thylakoids. The thylakoid fractions were incubated with 0.1 M Na_2CO_3 for 30 min on ice, followed by centrifugation at 80,000 g for 10 min to obtain the pellet (3, 7, 11) and the supernatant (4, 8, 12).

6.2.1.5 Localisation of the two forms of psNDPK2 *in vivo*

Furthermore, we ventured to investigate the two forms of psNDPK2 *in vivo*. For this purpose the PEG transformation method was applied. The inserts from the three constructs used for import studies (psNDPK2, psNDPK2-63RD and psNDPK2-74RD), were introduced at the N-terminus of the GFP/RFP in pOL-GFP and pOL-RFP vectors, respectively. *Arabidopsis thaliana*, pea or tobacco protoplasts were transformed with these constructs and their expression was observed under a microscope after 15-18 hours of incubation in the dark. All the three plant species showed similar expression behaviours with the constructs, therefore the protoplasts from *Arabidopsis thaliana* were used in the following experiments, if not mentioned otherwise. All the three constructs showed a similar phenotype in protoplasts transformed using PEG. psNDPK2-GFP/RFP were always localised in chloroplasts, as discretely distributed dots. Closer observation using confocal microscopy revealed that the dots appear to be associated with the thylakoids as can be seen in Fig. 36, where the red channel represents the chlorophyll autofluorescence and green the GFP signal. This kind of distribution is quite similar to that observed for mature chloroplast nucleoids (Sato *et al.* 1993). For psNDPK2-63RD-GFP and psNDPK2-74RD-RFP, the majority of the transformed protoplasts were as for the wild type but few showed discrepancy in being concentrated in the middle of the chloroplast as a blob or having fewer dots.

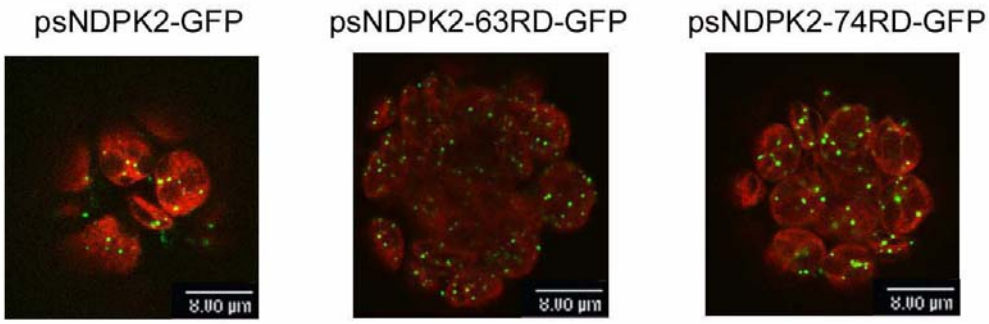

Figure 36. PEG transformation of the GFP/RFP fusion constructs for psNDPK2, psNDPK2-63RD and psNDPK2-74RD into isolated protoplasts. Red channel represents the chlorophyll autofluorescence, whereas the green is the GFP fluorescence.

In order to see any difference in the localisations of the two forms of psNDPK2, co-transformation of protoplasts with psNDPK2-63RD-GFP and psNDPK2-74RD-RFP was performed. Upon observance of the transformed protoplasts under the microscope, both forms were seen to occur at the same positions (Fig. 37). This could be observed by the overlap of the green fluorescence of psNDPK2-63RD-GFP and the red fluorescence emitted by the psNDPK2-74RD-RFP. The results from this experiment is in agreement with that of the *in vitro* import experiments where both forms of psNDPK2 were found to be localised in the same compartment of chloroplasts.

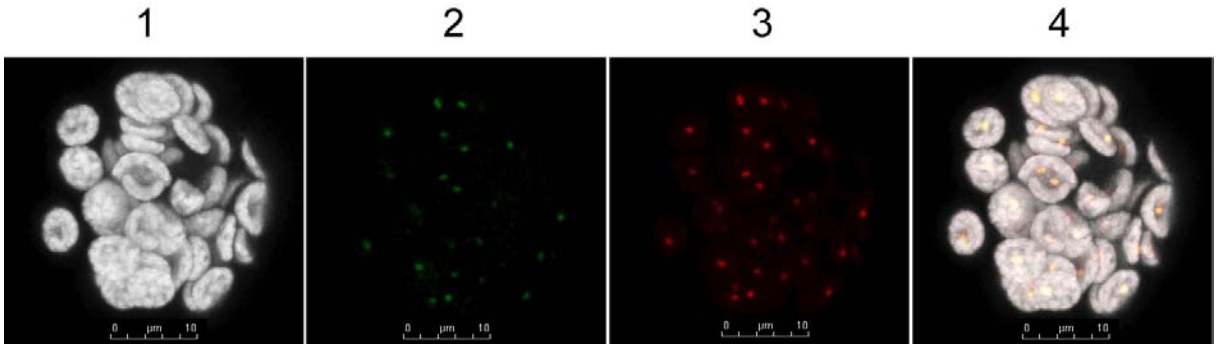

Figure 37. Co-localisation of psNDPK2-63RD-GFP and psNDPK2-74RD-RFP in isolated protoplasts. Isolated protoplasts were co-transformed with psNDPK2-63RD-GFP and psNDPK2-74RD-RFP constructs using the PEG-transformation method and viewed after 15-18 hours under a confocal microscope. (1) chlorophyll autofluorescence, (2) GFP fluorescence of psNDPK2-63RD-GFP, (3) RFP fluorescence of psNDPK2-74RD-RFP, (4) overlap of all the 3 channels.

6.2.1.6 The *in vivo* expression levels of the two forms of psNDPK2

Further, to find out whether the expression and proportion of the *in vitro* transcribed, translated and chloroplast imported forms of psNDPK2 are maintained at the level of the *in vivo* transcribed, translated and imported proteins in isolated protoplasts as well, the following experiment was conducted. The protoplasts transformed with psNDPK2-RFP/GFP, psNDPK2-63RD-GFP and psNDPK74RD-GFP/RFP were pelleted 18-24 hours after transformation. Western blot analysis using antibodies against GFP or Ds-RED was then performed. The results are presented in Fig. 38. The blots revealed that the two forms of the protein are also observed in *in vivo* experiments in similar proportions to that of the *in vitro* data. The two bands for psNDPK2-RFP and psNDPK2-77AT-RFP correspond to their theoretical molecular weights of 48.5 and 47.4 kD respectively, whereas psNDPK2-63RD-GFP and psNDPK2-74RD-RFP give rise to proteins of around 48.5 kD and 47.4 kD molecular weights, respectively (Fig. 38A and B). For the experiment presented in Fig. 38A, pea protoplasts were used whereas *Arabidopsis thaliana* protoplasts were used for the experiment depicted in Fig. 38B. Similarity of the bands obtained in pea and *Arabidopsis thaliana* protoplasts to the *in vitro* imported products (section 6.2.1.2) also indicates that the expression of this protein is independent of the plant species used for its expression.

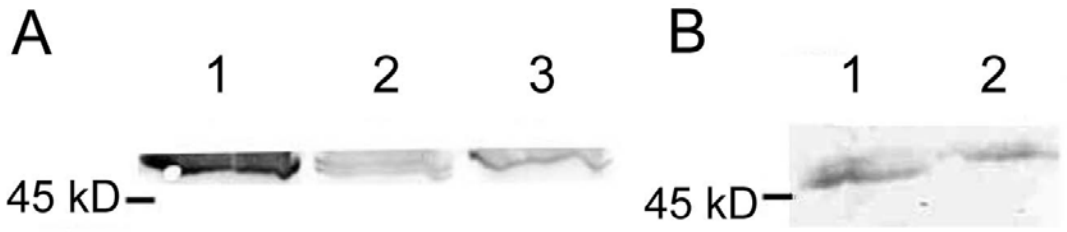

Figure 38. *In vivo* expression levels of the two forms of psNDPK2. **A**. Pea protoplasts were isolated from the leaves of 12-15 day old plants and transfected with psNDPK2-63RD-GFP (lane 1), psNDPK2-77TA-GFP (lane 2) and psNDPK2-74RD-GFP (lane 3). After incubation in dark for 24 hours, the protoplasts were pelleted, lysed with 4x Lammeli buffer and run on SDS-PAGE. It was followed by immunoblot analysis using the antibody against GFP. **B**. Protoplasts from the leaves of 3-4 week old plants of *Arabidopsis thaliana* were isolated and transformed with psNDPK2-RFP (lane 1) and psNDPK2-74-RFP (lane 2). It was followed by immunoblot analysis using anti-DsRED antibody.

All the above experiments indicated that the two forms of the pea psNDPK2 do not show any visible difference in their import behaviours, localisations and proportion of expressions *in vitro* as well as *in vivo*.

6.2.1.7 Intra-organellar localisation of psNDPK2 in chloroplasts

One aspect which emerged from the above study was that the fluorescence signal observed in chloroplasts after transient expression of GFP or RFP-fused psNDPK2 in protoplasts was in the form of discrete dots throughout the chloroplast, as mentioned earlier. Upon closer examination it was found that in most of the cases, these spots were located on the thylakoid membranes, facing the stroma (Fig. 36). This distribution of the psNDPK2 (Fig. 39A) strongly resembles that of the nucleoids in the mature chloroplasts (Fig. 39B) (Sato *et al.* 1993). In order to check for the possible nucleoid localisation of psNDPK2, three experimental procedures were followed.

1. Co-localisation of psNDPK2 with DAPI-stained nucleoids

Protoplasts were transformed with psNDPK2-RFP and then stained with DAPI as described in section 5.3.19. Upon comparing the fluorescence emitted by RFP-fused protein with that of the fluorescence emitted by DAPI-stained nucleoids, it was seen that the fluorescent labels of both samples overlapped (Fig. 39C), though this overlap was not absolute (Fig. 39D). It has been found to be in the range of 40% to 70% based upon three independent experiments. These results indicate an association of psNDPK2 with nucleoids. Their partial overlap also makes it likely that this assocaition is not exclusive and the psNDPK2 may bind to other complex or complexes as well, which needs to be further investigated.

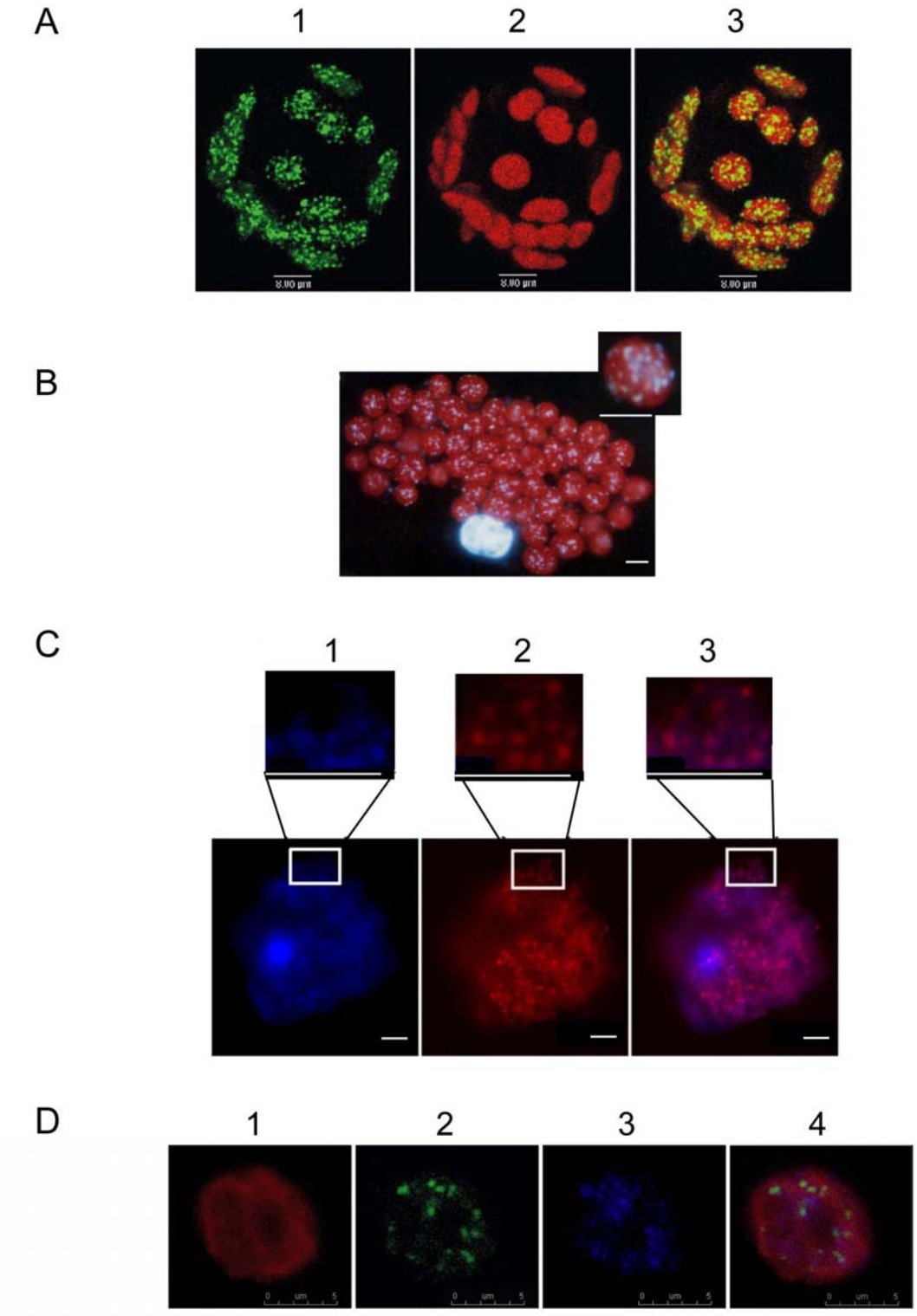

Figure 39. Localisation of psNDPK2-RFP fusion protein to plastid nucleoids. **A**. Localisation of psNDPK2-RFP in tobacco protoplasts. 1, RFP fluorescence from psNDPK2-RFP; 2, chlorophyll autofluorescence; 3, overlap of 1 and 2. **B**. Visual localisation of nucleoids in mature chloroplasts through DAPI staining of pea protoplasts as published by Sato *et al.*, in EMBO J. 1993 12(2): 555–561. Inset picture shows an enlarged chloroplast stained with DAPI. Bars represent 5 µm. **C** and **D**. psNDPK2-RFP was transiently expressed in isolated protoplasts, fixed and stained with DAPI and observed under a microscope at their respective wavelengths. Bars represent 5 µm. **C**. The lower row shows a single protoplast and the inset pictures above presents a magnified chloroplast in this protoplast. 1. DAPI-stained protoplast (blue); 2. psNDPK-RFP-expressing protoplast (red); 3. overlap of 1 and 2. **D**. A single chloroplast, 1. autofluorescence of chlorophyll (red); 2. fluorescence of RFP from the fusion protein psNDPK-RFP (green); 3. DAPI fluorescence (blue); 4. overlap of 1, 2, and 3.

2. Co-localisation of psNDPK2-RFP and APO1-GFP in chloroplasts

A recently identified nuclear-encoded factor, ACCUMULATION OF PHOTOSYSTEM ONE1 (APO1), required for the accumulation of PSI and other plastid-encoded and nuclear-encoded [4Fe-4S] cluster proteins of the chloroplast was shown to be associated with nucleoids (Amann *et al.* 2004). Upon co-transformation of psNDPK2-RFP and APO1-GFP, most of the distinct dots expressed in chloroplasts for both the constructs were found to overlap but there was still some diffuse APO1-GFP in the stroma which did not correspond with the psNDPK2-RFP signal (Fig. 40). These results are in accordance with those reported by Amann and co-workers (2001), where the fluorescence signal for APO1-GFP was shown to be throughout the stroma but much of it was reported as distinct spots within chloroplasts. This co-localisation of psNDPK2 and APO1 in the chloroplast further strengthens the notion of the association of psNDPK2 with nucleoids.

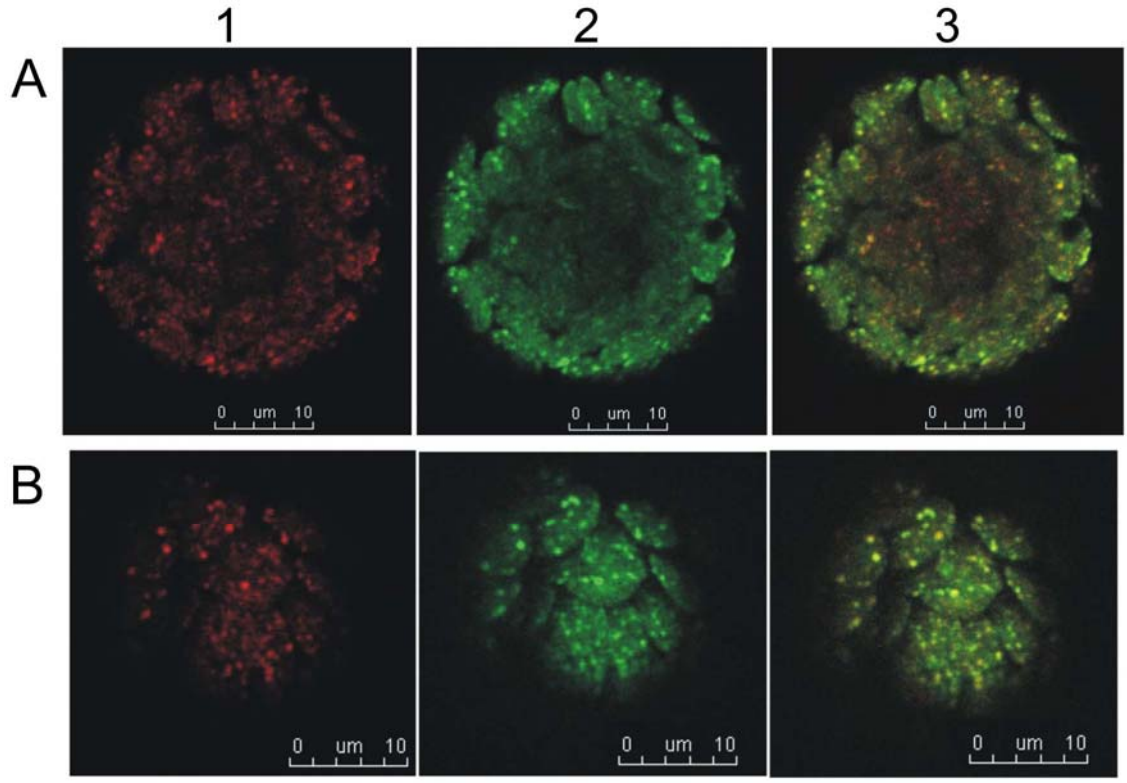

Figure 40. Co-localisation of psNDPK2-RFP and APO1-GFP in chloroplasts. Figures A and B represent the same protoplast in two different focal planes as observed under a confocal microscope. Lane 1 shows the protoplast expressing the psNDPK2-RFP protein. Lane 2 represents APO1-GFP expressed in the same protoplast. Lane 3 is the overlap of the images 1 and 2.

3. Nucleoid isolation from chloroplasts

Another approach applied to further analyse the association of psNDPK2 with the nucleoids was to isolate the nucleoids from isolated pea chloroplasts followed by their immunoblot analysis with antibody against NDPK2. The method used for the isolation of nucleoids from chloroplasts is described in section 5.3.21. The purity of the nucleoids

obtained was tested by staining 1 µl of the nucleoids with DAPI and observing them under a confocal microscope (Fig. 41). In addition, the different fractions from the nucleoid isolation procedure were subjected to immunoblot analysis using antibodies against SiR (a nucleoid protein), FNR, Tic22 and psNDPK2 (Fig. 42). FNR and Tic22 antibodies were used to exclude contamination of other chloroplast proteins in the purified nucleoid fraction. Interestingly, no psNDPK2 was found in the fraction containing the purified nucleoids. It was found to detatch after the first high speed centrifugation step (S2) and none was present with the isolated nucleoids.

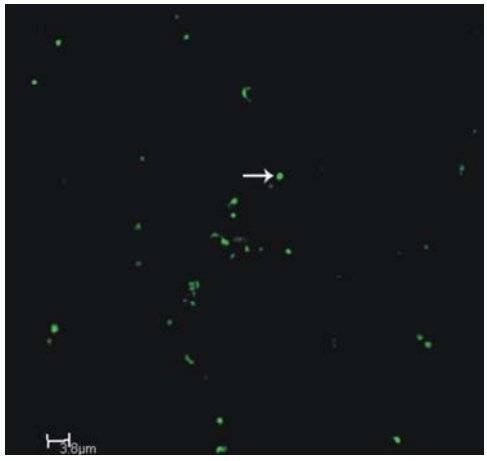

Figure 41. Epifluorescence microscopy of 1 µl of the purified nucleoid fraction shown in lane 'S5' of Fig. 42 after DAPI staining.

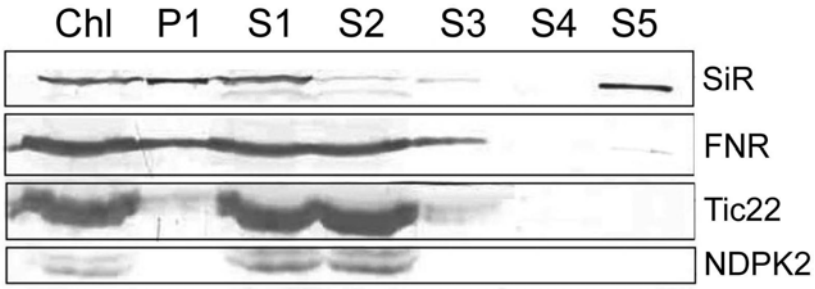

Figure 42. Binding of psNDPK2 to the nucleoids. Immunoblot analysis of the different fractions of the nucleoid isolation procedure with the nucleoid protein ferredoxin:sulfite reductase (SiR, 70 kD), the stromal protein FNR (36 kD), the chloroplast inner envelope protein Tic22 and NDPK2 (section 5.3.21).'Chl' represents the total chloroplast extract; P1, pellet fraction obtained after lysis of chloroplasts and first short centrifugation step; S1, first supernatant; S2, second supernatant; S3, supernatant obtained after third centrifugation; S4, supernatant after fourth centrifugation; 'S5', purified nucleoid fraction.

The results from these three experimental approaches suggest that although psNDPK2 protein seems to be associated with nucleoids, it does not bind tightly to them. It is also likely that it is indirectly associated with nucleoids by its interaction with one or more other proteins of the nucleoids.

6.2.1.8 Investigation into the proposed DNA binding sites in psNDPK2

NM23-H2, a presumed regulator of tumor metastasis in humans, is a hexameric protein with both enzymatic (NDP kinase) and regulatory (transcriptional activation) activity. Within the sequence of this protein, Postel and coworkers (1996), found three amino acids, among 30 possibilities, to be critical for the binding of NM23-H2 to DNA. All the three DNA-binding defective mutant proteins are enzymatically active and appear to be stable hexamers, suggesting that separate functional domains exist for enzyme catalysis and DNA binding (Postel *et al.* 1996). The crystal structure of NM23-H2 has been deciphered by Webb et al. (1995) according to which NDPK2 exists as a hexamer having a 3-fold symmetry with 2 layers. Based on this, the three above mentioned DNA-binding amino acids were marked in the structure using the PyMPOL program (Fig. 43A). Two out of these three DNA-binding sites were found to be conserved in the plant species (Zimmermann *et al.* 1999). Since the alignment studies, discussed later on in section 6.2.2, show a high similarity between psNDPK2 and atNDPK2, and the crystal structure of *Arabidopsis thaliana* NDPK2 is known (Im et al., 2004), we constructed a similar figure for the atNDPK2 as for the human NM23-H2 (Fig. 43B). In the two structures, the positions of the marked residues were seen to be similar in both cases.

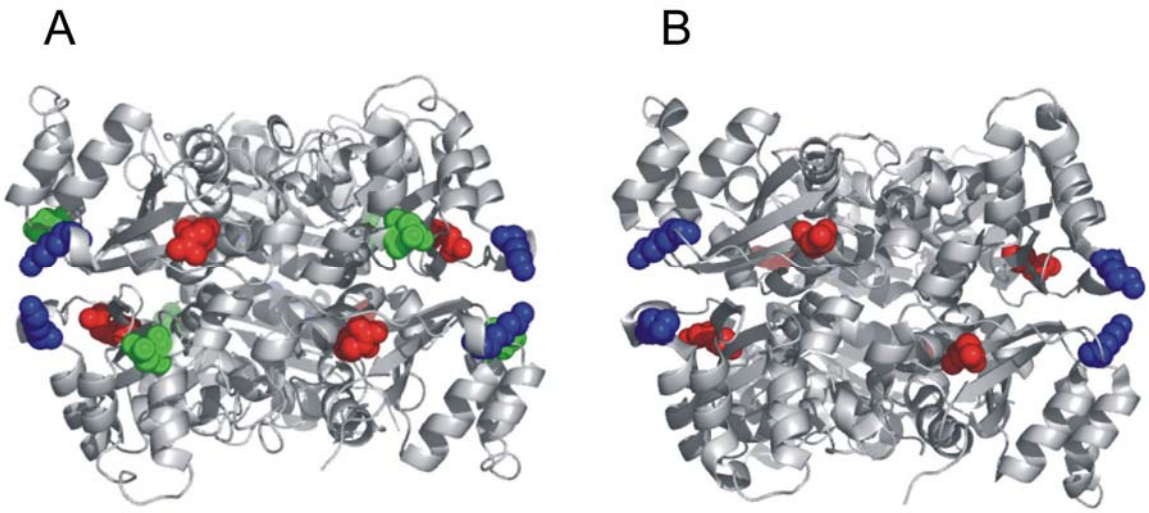

Figure 43. Ribbon diagrams of human NM23-H2 and atNDPK2. **A**. The structure of human NM23-H2 with the three amino acids, Arg-34, Asn-69 and Lys-135 which were found to be critical for DNA binding marked with red, blue and green colours, respectively. **B**. Structure of *Arabidopsis thaliana* NDPK2 with the two conserved amino acids Lys-113 and Lys-214 marked in blue and red, respectively.

In order to investigate the possible DNA-binding of psNDPK2 at these positions, two experimental approaches were followed:

1. Mutagenesis of the putative/proposed DNA-binding sites in psNDPK2

Point mutations were done for the two proposed DNA-binding sites in psNDPK2. Accordingly, the lysines at positions 112 and 213 of psNDPK2-RFP were changed to an arginine and histidine, respectively. The choice of the amino acid substitution was in accordance to the residues found to block the DNA binding in the previous study (Postel et al., 1996). If these are really the sites of binding for psNDPK2 with the chloroplast DNA, as is the case for the human NM23-H2 homologue with nuclear DNA, then the expression of the mutated constructs, psNDPK2-112KA-RFP and psNDPK2-213KH-RFP, upon being introduced into isolated protoplasts would probably show a difference in their distribution as compared to the distinct dots seen for the wild type psNDPK2-RFP. For this purpose, these constructs were transformed into protoplasts using the PEG transfromation method. Upon the observance of the transformed protoplasts under a confocal microscope, no difference in the distribution of the mutated proteins was observed from that of the wild type 'dotted' phenotype (Fig. 44). Further, the plasmids containing the psNDPK2-112KA-RFP and psNDPK2-213KH-RFP inserts were co-transformed with APO1-GFP construct into isolated protoplasts. This was done to find any difference in the localisation of the dots obtained from the mutated protein fusions and APO1-GFP. No difference in the distribution of the proteins was observed after such co-transfections (Fig. 45A and B).

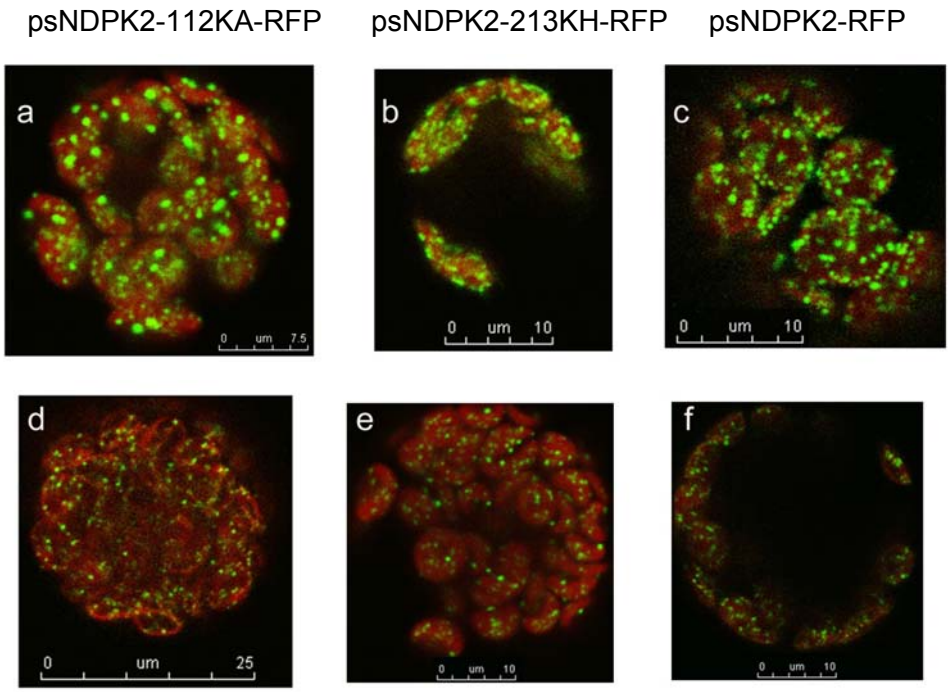

Figure 44. Mutagenesis of the putative DNA-binding sites in psNDPK2. psNDPK2-112KA-RFP (a and d), psNDPK2-213KH-RFP (b and e) and psNDPK2 (c and f) were transformed into isolated protoplasts and observed under a confocal microscope.

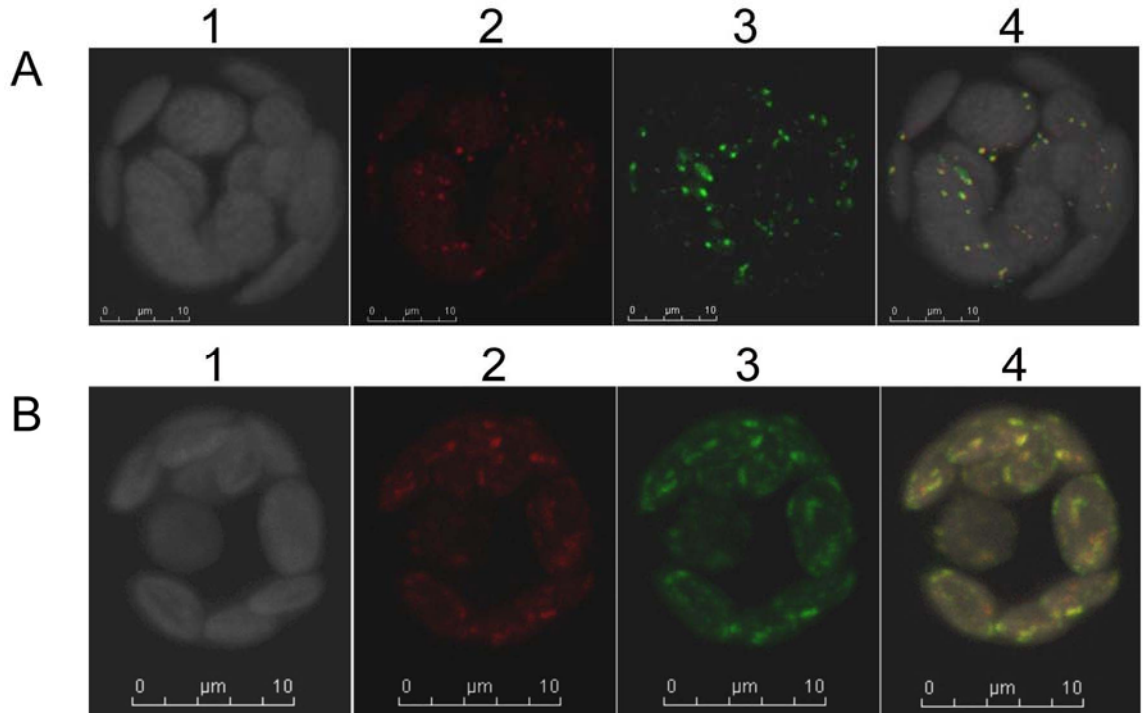

Figure 45. Co-transformations of psNDPK2-112KA-RFP (A) and psNDPK2-213KH-RFP (B) with APO1-GFP. 1. Chlorophyll autofluorescence (grey). 2. Red fluorescence from psNDPK2-112KA-RFP (Fig. A) and psNDPK2-213KH-RFP (in Fig. B) 3. Green fluorescence emitted by APO1-GFP. 4. The overlay image of all the three channels.

2. Binding assay to check for a possible DNA-binding of psNDPK2

In another approach to find out whether the psNDPK2 binds to the chloroplast DNA, the chloroplast DNA was purified from isolated pea chloroplasts, as described in section 5.1.5. The purified DNA was then restricted with XhoI and SalI enzymes. The restricted fragments were ligated to linkers of about 35 base pairs in length. On the other hand, the psNDPK2 protein was overexpressed in *E. coli* cells followed by its purification over Ni-NTA matrix. The soluble protein was incubated with the linker-attached chloroplast DNA fragments. After the incubation step, the protein was immunoprecipitated on Protein A sepharose by using the antibody against psNDPK2. This was followed by performing a PCR reaction using the elution obtained after immunoprecipitation as the source of the template and the linkers as the source of primers. One band was observed on the agarose gel after loading the PCR product on it. Upon sequencing of the band obtained after the PCR reaction, it was found to consist of the linkers ligated to each other. To test for the possible attachment of DNA to Protein A sepharose, a plasmid DNA was incubated with Protein A sepharose followed by its thorough washing from the sepharose matrix. Then the elution step was performed. The PCR reaction carried out with the eluate by using plasmid-specific primers revealed a band of the size of the sequence between the two primers. From this, it could be concluded, that Protein A sepharose itself is capable of binding to DNA. Therefore this approach could not give the required proof for the DNA-binding capability of psNDPK2.

6.2.2 Localisation of atNDPK2 within the plant cell

During our study of the NDPK2 enzyme we came across two conflicting views prevailing in the literature regarding its localisation within the plant cell. For pea, spinach and *Brassica campestris*, it was reported to be in chloroplasts whereas the NDPK2 from *Arabidopsis thaliana* was shown to be in the cytosol and nucleus (NDPK2, Choi *et al.*, 1999; NDPK Ia, Zimmermann *et al.* 1999). NDPK Ia, which was reported to be another protein in the original publication (Zimmermann *et al.* 1999), was recently (09.June.2006) re-annotated to be NDPK2 in the NCBI protein database. Since for all the other plant species studied till date, the NDPK2 isoform was found to be in the chloroplast, the investigation of the subcellular localisation of the NDPK2 protein of *Arabidopsis thaliana* was undertaken in this study.

6.2.2.1 Phylogenetic analysis of plant NDPK proteins

The sequence of NDPK has been reported to be highly conserved in both eukaryotes and prokaryotes. To study the different evolutionary groups of NDPKs and to place *Arabidopsis thaliana* NDPK2 among them, an alignment with NDPK isoforms from *Arabidopsis thaliana*, *Brassica rapa*, spinach and pea was performed using ClustalX program (Fig. 46). The sequences were retrieved from the NCBI protein database.

Upon calculation of the percentage of identity between the mature protein sequence of atNDPK2 with those of NDPK2 from pea, spinach and *Brassica rapa* using the BELVU program (Sonnhammer, 1999), it was found that atNDPK2 shows highest identity with *Brassica rapa* (94%), 85.5% with spinach and 81.3% with pea NDPK2. The homology among the four plant species studied is between 78.3% to 94% with the mean value of 84%. The NDPK isoforms form three groups, out of which the NDPK2 and NDPK3 contain a distinct predicted transit peptide at their N-termini. The active site histidine of the NDPKs is highly conserved (His-197 in atNDPK2).

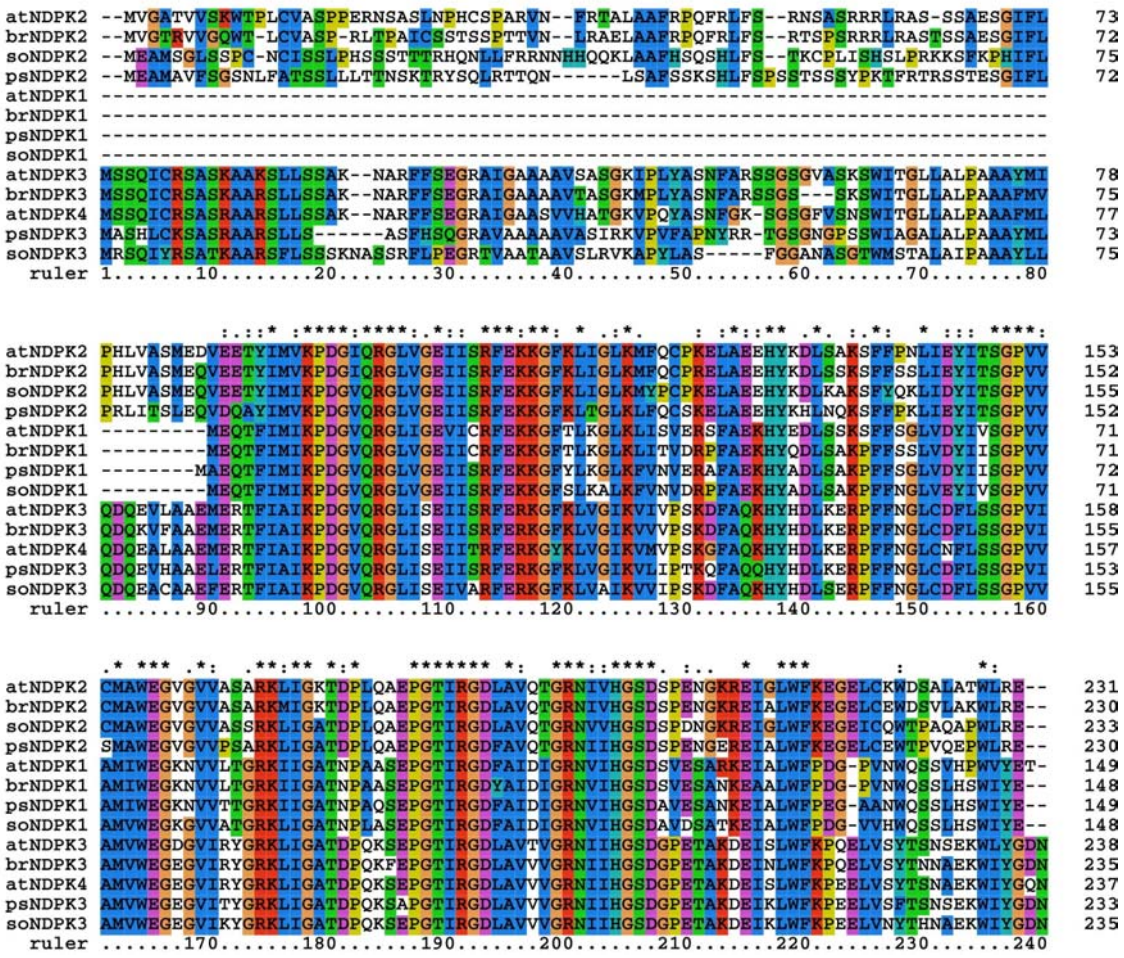

Figure 46. Sequence alignments of *Arabidopsis thaliana*, *Brassica rapa*, spinach and pea NDPK isoforms using the ClustalX program (Thompson et al., 1997). The sequences were obtained from GenBank as follows: psNDPK1 (P47922); psNDPK2 (P47923); psNDPK3 (AAF08537); atNDPK2 (O64903); atNDPK4 (Q8LAH8); atNDPK3 (O49203); atNDPK1 (P39207); soNDPK3 (P81766); soNDPK1 (Q02254); soNDPK2 (Q01402); soNDPK4 (Q8RXA8); brNDPK3 (BAB86842); brNDPK1 (BAB86841); brNDPK2 (BAC05487), where 'ps' is *Pisum sativum*, 'at' is *Arabidopsis thaliana*, 'so' is *Spinacia oleracea* and 'br' is *Brassica rapa*. '*' represents fully conserved, ':' highly conserved and '.' conserved residues.

6.2.2.2 Targeting of atNDPK2 into isolated chloroplasts

To biochemically check for the chloroplastic localisation of the atNDPK2, *in vitro* import assays were carried out as done previously for the psNDPK2. *In vitro* translation of the atNDPK2-pBluescript clone (see section 5.1.3.3) gave rise to mainly a single translation product of around 25 kD (Fig. 47, lane 1). The size of the precursor protein corresponded well with the theoretical value of 25.5 kD calculated from the deduced amino acid sequence. After incubation of the translated protein with isolated pea chloroplasts in import mix, the presence of approximately 18 kD and 17 kD bands were observed (Fig. 47, lane 3). Upon thermolysin treatment of chloroplasts after the import reaction, the two bands were found to be thermolysin protected (Fig. 47, lane 2), indicating that the atNDPK2 is truly imported into chloroplasts. The occurrence of two bands is similar to that observed for psNDPK2 which has already been discussed in detail in section 6.2.1.

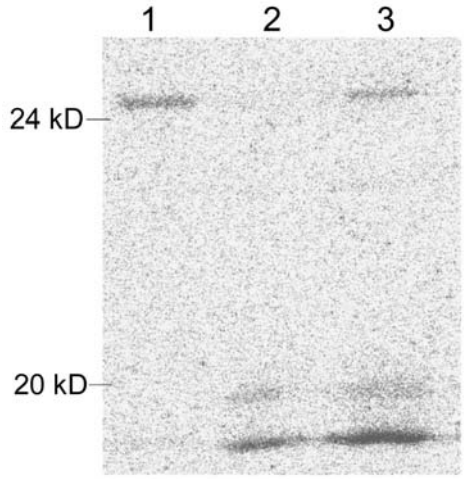

Figure 47. *In vitro* import of atNDPK2. *In vitro* translated atNDPK2 was imported into isolated chloroplasts. Lane 1 shows one-tenth of the translated protein used in the import reactions; lane 2, *in vitro* imported atNDPK2 followed by thermolysin treatment of chloroplasts; lane 3, import reaction without thermolysin treatment.

6.2.2.3 Transient expression of atNDPK into protoplasts

In order to localise atNDPK2 *in vivo* within the plant cell, we cloned the full length cDNA of atNDPK2 in pOL-GFP vector. This construct was transfected into isolated *Arabidopsis thaliana* protoplasts using the PEG transformation method as described in section 5.3.11. The expression of the GFP-tagged proteins was observed under a confocal microscope (Fig. 48). According to the images obtained, the GFP fluorescence emitted by the atNDPK2-GFP protein was found to be from chloroplasts. No fluorescence signal was observed in the cytosol or the nucleus. The distribution of this protein occurs as distinct dots throughout the chloroplast as was observed for the psNDPK2-RFP protein (section 6.2.1.3). On closer examination, these dots seemed to be in the stroma, but in close association with the thylakoids.

Further, the N-terminal deletion for atNDPK2 which lacked the transit peptide of the protein was constructed with an RFP tag at its C-terminus (atNDPK2-Δ1-79-RFP). Upon its transformation into isolated protoplasts, the distribution of this protein was found to differ from that of the full-length. In this case, the expressed protein was found to be in the cytosol only (Fig. 49).

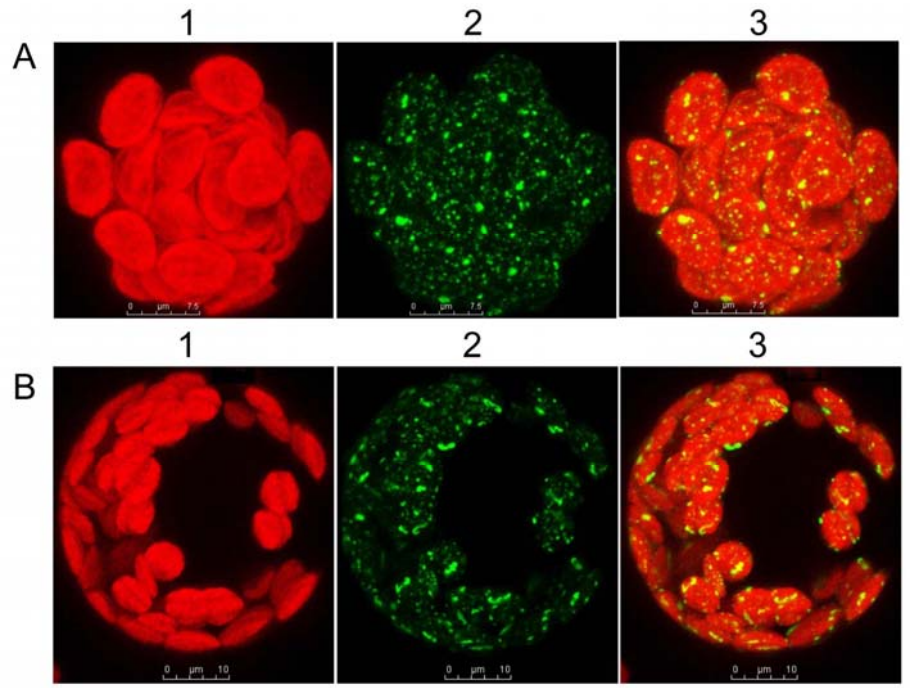

Figure 48. Localisation of atNDPK2-GFP to chloroplasts. The atNDPK2 was inserted into the pOL-GFP vector to generate the construct atNDPK2-GFP, containing a C-terminal GFP fusion. This plasmid was introduced into protoplasts obtained from the leaves of 3-4 week old *Arabidopsis thaliana* plants by polyethylene glycol-mediated transformation. The transformed protoplasts were examined after 14-18 hours under a confocal microscope. A and B represent the same protoplast in two focal planes. (1) Chlorophyll autofluorescence, (2) Expression of atNDPK2-GFP (3) Overlap of the chlorophyll autofluorescence and the atNDPK-GFP protein expressed in a protoplast.

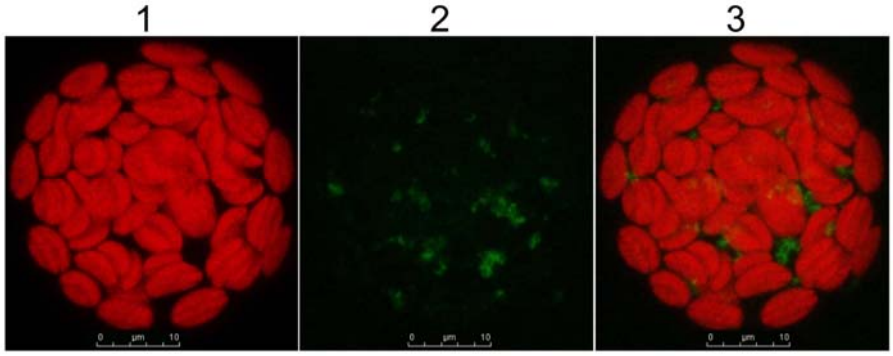

Figure 49. Localisation of atNDPK2-Δ1-79-RFP in isolated protoplasts. The deletion construct lacking the transit peptide of atNDPK2 was generated by PCR and inserted into pOL-RFP to give rise to atNDPK2-Δ1-79-RFP, having a C-terminal RFP fusion. Protoplasts isolated from the leaves of 3-4 week old *Arabidopsis thaliana* plants were transformed with it and observed under a confocal microscope after 14-18 hours of transformation. (1) Chlorophyll autofluorescence, (2) atNDPK2-Δ1-79-RFP fluorescence, (3) Overlay of the chlorophyll autofluorescence and the RFP fluorescence channels.

6.2.2.4 Distribution of atNDPK isoforms in the cell

Another approach applied to address the problem of the localisation of atNDPK2 within the plant cell was through immunoblot analysis of the cytosol and the rest of the cell contents using *Arabidopsis thaliana* protoplasts. Protoplasts were isolated from 3-4 weeks old *Arabidopsis thaliana* plants as described in section 5.3.14 using the gradient method to obtain only intact protoplasts. These were used to obtain the cytosolic and the other cell-

fractions followed by western blot analysis with the antibodies against the chloroplast nucleoid associated protein, ferredoxin:sulfite reductase (SiR); cytosolic 14-3-3 and the antibody against NDPK2 (Fig. 50). Immunoblot against anti-SiR showed a prominent band at the expected 70 kD size in the pellet fraction and a quite faint one in the cytosolic fraction. This was due to the fact that using the procedure followed here, it is not possible to obtain entirely pure cytosol. A certain amount of contamination is expected from the soluble fractions of other compartments of the cell as well. A number of bands were observed for the 14-3-3 proteins, both in the pellet and the cytosolic fraction. The reason for this could be that at least 15 putative 14-3-3 proteins have been reported for *Arabidopsis thaliana* (Rosenquist *et al*., 2001) of which at least four are supposedly in chloroplasts (Sehnke *et al*., 2000). Therefore the antibody against the cytosolic 14-3-3 cross-reacted with those of the other compartmental forms of the protein as well. But the difference between the number of bands and intensities in the two fractions is quite distinct. The western blot analysis carried out against the atNDPK2 antibody showed two bands in the pellet fraction at around 18 kD and 17 kD, and a lower band at around 16 kD in the cytosol. According to the NCBI database, four isoforms of NDPK have been reported. Their mature forms are reported to be 16.5 (NDPK1), 18.5 kD (NDPK2) and 17 kD (NDPK3 and NDPK4). A band observed at around 29 kD in the pellet fraction was unspecific because it was also observed with the pre-immune serum of anti-NDPK2. The results from this experiment indicate that there exists only one isoform of NDPK in the cytosol which corresponds to the 16 kD NDPK1 isoform and two other isoforms in the rest of the cell.

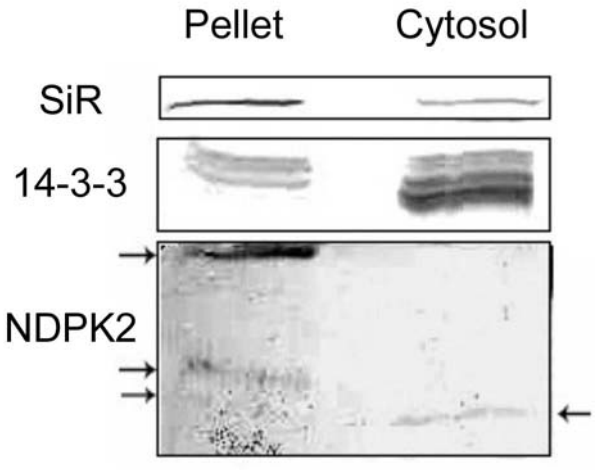

Figure 50. Localisation of the different isoforms of NDPK2 in *Arabidopsis thaliana* protoplasts through immunoblot analysis. Intact protoplasts were isolated followed by their fractionation into the pellet and the cytosolic fractions. Immunoprecipitation with these fractions was performed using the antibodies against chloroplastidal SiR protein, cytosolic 14-3-3 and NDPK2.

All the experiments conducted to find out the location of the atNDPK2 in *Arabidopsis thaliana* indicated to its being present within chloroplasts. Despite these evidences, we ventured to speculate if it is possible for atNDPK2 to be present in the cytosol or the nucleus

in addition to being in chloroplasts. To examine this idea, we carried out two experiments that are described below.

6.2.2.5 atNDPK2 is not re-translocated into the cytosol after import into chloroplasts

A possibility which was considered for the reported presence of atNDPK2 in the cytosol by Choi *et al*. (1999), and Zimmermann *et al*., (1999) was that maybe after the import of atNDPK2 into chloroplasts, it might be re-translocated back to the cytosol. For this purpose, first of all, the *in vitro* translated atNDPK2 was imported into isolated chloroplasts. Chloroplasts were then pelleted and both the supernatant and the pellet were loaded onto the SDS-PAGE (Fig. 51, lanes 2 and 3). This was done as a control to check that the supernatant contained only the translation band consisting of the non-imported protein and not the truncated imported protein. In the next experiment, the *in vitro* translated atNDPK2 was allowed to bind to chloroplasts on ice for five minutes. Chloroplasts were then washed to remove the unbound protein. The chloroplast-bound proteins were then subjected to import reaction. After incubation, chloroplasts were pelleted and both the supernatant and the pellet were loaded onto a SDS-PAGE gel. If the imported atNDPK2 was redirected back to the cytosol after import, it would be present in the supernatant. But no protein was observed in the supernatant fraction (Fig. 51, lane 5). All the protein was localised inside chloroplasts (Fig. 51, lane 4). As a control, these two experiments were also done with the *in vitro* translated pSSU protein. The bands obtained for pSSU were similar to those observed for atNDPKs (autoradiogram for pSSU not shown).

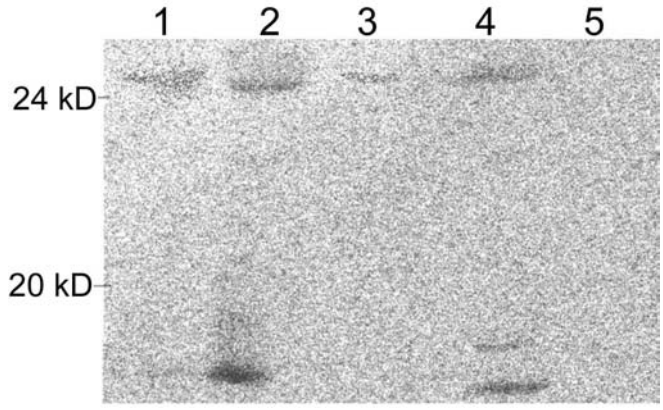

Figure 51. Relocation of atNDPK2 into cytosol. Lane 1, one-tenth of the translation product taken in each import reaction. Lanes 2 and 3: atNDPK2 was allowed to be imported into isolated chloroplasts. Chloroplasts were then pelleted and supernatant was collected. Lane 2 represents the autoradiogram of the pellet whereas lane 3 is the supernatant. Lanes 4 and 5: *In vitro* translated atNDPK2 protein was added to the usual import assay and incubated on ice for 5 minutes. The intact chloroplasts were then isolated over 40% percoll. These protein-bound chloroplasts were then subjected to normal import reaction. Chloroplasts were pelleted again and both the pellet (lane 4) and the supernatant (lane 5) were loaded onto SDS PAGE, followed by autoradiography.

6.2.2.6 A single mRNA is observed for atNDPK2 in *Arabidopsis thaliana*

Next, we performed RT- and RACE-PCRs to check for the probability of the occurence of more than one form of atNDPK2 mRNA in the cell which could arise by alternative splicing. The proteins that could be transcribed by different forms of mRNA might indicate their presence in different compartments. For this purpose, mRNA was isolated from 7 or 21 days old *Arabidopsis thaliana* plants and it was reverse transcribed to obtain cDNA. Upon performing RT-PCR with this cDNA using the forward primer at the 5'-UTR of atNDPK2 and the reverse primer in its third exon (Fig. 52), it was found to give rise to only one PCR product of around 420 bps (Fig. 53). This band was sequenced and was found to be a part of the full length protein starting with its transit peptide. RACE-PCR was then done using mRNA from 21 days of *Arabidopsis thaliana* plants. In this case, touch-down PCR was performed using the universal primer provided in the kit and the primer in the seventh exon of atNDPK2. In this case also, only one band (around 750 bp) was obtained which upon sequencing was found to be part of the full length sequence including the N-terminal transit peptide (Fig. 54). These results indicated the presence of only one form of mRNA in the *Arabidopsis thaliana* plants for the atNDPK2.

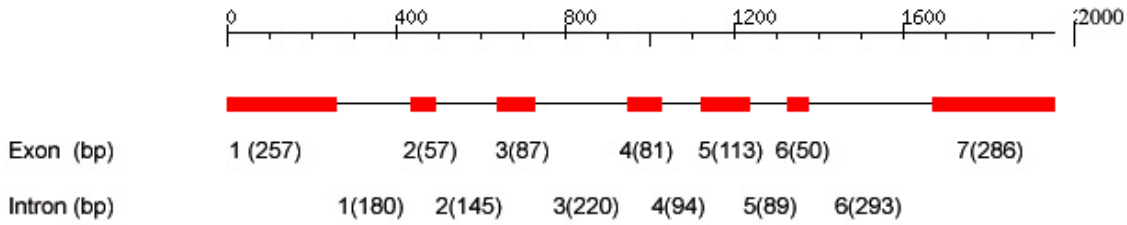

Figure 52. Schematic representation of the Intron/Exon structure of atNDPK2 in nucleotide coordinates relative to the start of the gene. The black blocks represent the exons while the thin lines are the introns. Below the figure the numbers of the exon or the intron are given followed by their respective lengths in base pairs (bp).

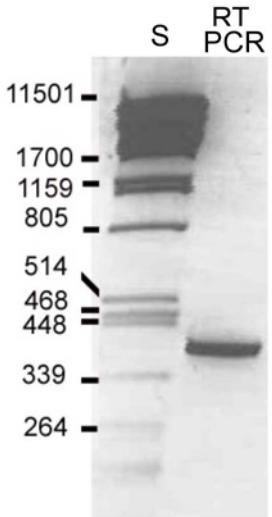

Figure 53. RT-PCR. The first lane from left represents the standard marker obtained by the restriction of lambda DNA with the Pst I restriction enzyme, and the second lane containes the PCR product obtained by using the cDNA obtained from the reverse transcribed mRNA of 21 days old *Arabidopsis thaliana* plants. The forward primer used was in the 5'- UTR and the reverse primer was in the third exon of atNDPK2.

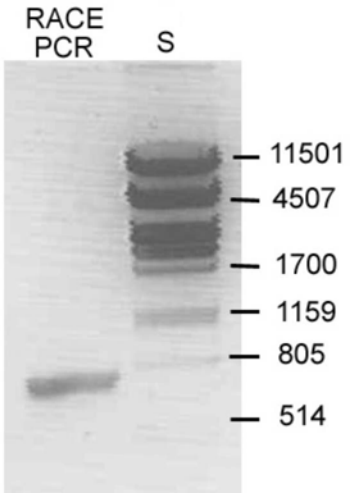

Figure 54. RACE-PCR. The cDNA for the 5' RACE-PCR was obtained from poly-A mRNA using the BD SMART RACE kit (Clontech). The forward primer used for the purpose was the UPM (Universal Primer A Mix) supplied in the kit and the reverse primer was gene specific derived from the seventh exon of atNDPK2 sequence. The lane marked as "S" is the standard marker.

7. Discussion

The transit peptides of chloroplast preproteins contain the key to the transport of nuclear-encoded proteins into chloroplasts. They are considered to be sufficient for this transfer to take place. Despite this essential feature, very little is known about them. In the present work, a study was undertaken to unravel more of the mysteries surrounding them. Plastidal transit sequences are very variable in length, ranging from 20 to more than 120 residues. They also display an immense variation in their primary amino acid sequence (von Heijne et al., 1989; Schein et al. 2001). An important feature specific to chloroplast transit sequences is a phosphorylation site consisting of a consensus motif that was recognised from the study conducted on the transit peptides of OE23 and OE33 from pea and pSSU from tobacco (Waegemann and Soll, 1996) and further confirmed by a study of *Chlamydomonas* pSSU (Su *et al*., 2001). The phosphorylation site was found to be at a serine or a threonine residue. May and Soll (2000) identified a 14-3-3 protein dimer, Hsp70 and possibly other proteins to form a guidance complex with which the interaction of the preprotein is strongly enhanced by the phosphorylation at its transit peptide. Our present knowledge of transit sequences is based mainly on *in vitro* experiments carried out using the transit peptides of only a small set of plastidal proteins, e.g. pSSU, pFdI (precursor proteins of ferredoxin isoprotein I). Therefore, we chose three chloroplast proteins from *Arabidopsis thaliana* (APC1, HCF136, and CAO) and a pea protein (LHCP), and tried to find their sites of phosphorylation and further attempted to study the effect of this phosphorylation upon the import process *in vivo*.

APC1 comprises of 13 serines and no threonine in its predicted transit peptide. Upon the mutation of all of these serines to an alanine, one at a time, all the mutant forms were found to be phosphorylated. This result indicated that either all the serines in the transit peptide of APC1 are phosphorylated or the site of phosphorylation shifts to another serine when the actual site of phosphorylation is mutated to an alanine. The former seems to be unlikely as it has been shown for pSSU, pOE23 and pOE33 that they are phosphorylated at only one serine in their transit peptides (Waegemann and Soll, 1996). Further mutations and amino acid analysis of the phosphorylated residue revealed that the serine at position 25 is phosphorylated, but it was found that one or more other serines are also phosphorylated at the same time. So the latter presumption of the shifting of the phosphorylation site also does not hold valid in this case because the phosphorylation of the Ser-25 and another serine residue occurs at the same time. This was also found to be the case for the LHCP from pea. Every mutation of a serine to an alanine led to the phosphorylation of pea LHCP transit peptide and no single amino acid residue could be identified to be the site of phosphorylation in this case. The reason for more than one site of phosphorylation for these two proteins is not known at present and needs further investigations.

In the case of the HCF136 protein, the site of phosphorylation was found to be either the serine at position 52 or 54. Since these two serines were found to be so close to each other, only one construct with the substitution from serines to threonines at positions 52 and 54 was made and tested for its ability to be phosphorylated. This double mutant was found to be phosphorylated but with a much lower intensity as compared to its wild type protein. The reason for this could be that the threonines are bigger in size as compared to serines and the presence of two threonines, at positions 52 and 54, may have caused steric hindrance in the efficient binding of the kinase, required for the phosphorylation to occur. In case of pSSU, it had been shown that the serine at position 34 was the site of phosphorylation but the serine at position 31 was suggested to function in the recognition or binding of the kinase (Waegemann and Soll, 1996). This could also be the case for HCF136.

Upon analysing the transit peptides of the above mentioned proteins with respect to their homology with the proposed phosphorylation motif (P/G)X_n(R/K)X_n(S/T)X_n(S*/T*), it was found that in the case of APC1, the serine at position 25 comes closest to resembling the site of phosphorylation (P S L S A D S S S L S*), although the motif in this case is not completely conserved. In the case of HCF136, the motif was found to be present within the transit peptide as: P R F L S Q R I S*. According to this motif, the site of phosphorylation should be the serine at position 27. But our mutagenesis approach revealed that the serines found to be phosphorylated in its transit peptide were at positions 52/54. The amino acids preceding these sites comprised of either prolines or serines and a lysine, P P P S P S P S S S S S S L S* F S*. In the proposed motif, P/G comprised the turn-promoting residue, followed by a spacer, a basic amino acid (R/K), a hydroxylated residue (S/T), a spacer and the actual phosphoacceptor amino acid (S*/T*) (Waegemann and Soll, 1996). From the site of phosphorylation found for APC1 and HCF136 it can be seen that the presence of the turn-promoting residue, spacers and a hydroxylated residue are common, but the basic amino acid in between them is conspicuous by its absence. These observations indicate that the occurance of phosphorylation at a certain serine or threonine may be dependent upon the secondary structure of the protein. In addition, the phosphorylation of transit peptides at serines/ threonines other than the ones from the proposed phosphorylation motif, lead us to propose that other yet-unknown chaperone molecules or cytosolic factors may also be involved in the recognition and guidance of preproteins to the chloroplast surface. This can also be concluded from the results obtained in the present work, where it was shown that the phosphorylation of more than one serine at the same time can take place, where different factors may recognise different sites of phosphorylation. Moreover, the CAO protein, in contrast to the other proteins studied, was not found to be phosphorylated at all in the *in vitro* phosphorylation assay although its transit peptide contained a clear phosphorylation motif.

This result led us to propose that not all the nuclear-encoded preproteins directed to chloroplasts share the ability to be phosphorylated by a cytosolic kinase *in vitro*.

Thus the analysis of the sites of phosphorylation for the three *Arabidopsis thaliana* proteins, APC1, HCF136 and CAO, and LHCP from pea revealed that not all preproteins directed to the chloroplast are phosphorylated in their transit peptides (CAO); the proteins that can be phosphorylated may not contain the proposed phosphorylation motif (APC1, LHCP); and even if they contain the exact phosphorylation motif, they may be phosphorylated at another site (HCF136). In addition, more than one site of phosphorylation may exist within the transit peptide and these may be phosphorylated at the same time (APC1 and LHCP).

In the earlier publication (Waegemann and Soll, 1996) it was reported that the non-phosphorylated pSSU protein was also imported into chloroplasts, indicating that phosphorylation of the transit peptide is not a prerequisite for the translocation of proteins *in vitro*. All the data available till now regarding the phosphorylation of preproteins have been obtained through *in vitro* studies. It is possible that this phosphorylation observed with recombinant proteins in an *in vitro* assay has no significance on the import of proteins in the plant cell. Therefore, it was endeavoured to study the significance of phosphorylation of preproteins upon the import process *in vivo*.

GFP-fused constructs of the wild type and the non-phosphorylatable mutant forms of pSSU, pOE23 and pSSU-mLHCP, when transfected into isolated protoplasts, were found similarly in chloroplasts. These experiments confirmed the previously reported *in vitro* results that the targeting of mutated transit peptides to chloroplasts is not affected *in vivo* as well. In a similar study conducted by Nakrieko *et al*. (2004) the transit peptides of pSSU from tobacco and pea as well as cyteinyl-tRNA and histidyl-tRNA from *Arabidopsis thaliana* were fused to GFP. The putative phosphorylation site in the transit peptide of these proteins was determined by the authors by comparing the transit peptide sequences with the consensus motif for phosphorylation and mutating them to alanines. No controls were made to check whether the phosphorylation sites were really blocked by these mutations or not. Our efforts at finding the site of phosphorylation in *Arabidopsis thaliana* proteins revealed that the presence of this motif does not necessarily lead to the phosphorylation exclusively at the predicted residue.

Transient expression of proteins is usually analysed 12-18 hours after transfection which represents a steady-state-level of translation and import. In order to observe protein expression and transport on a real-time basis, we decided to apply the technique of microinjecting single protoplasts with mRNA/DNA. A number of studies have been conducted which have utilised the microinjection technique for the transformation of protoplasts. Almost

all of them applied this technique for the transfer of the DNA and then the propagation of these protoplasts to obtain transformed plants (Schnorf *et al*., 1991). Our purpose of using the microinjection technique diverged from this approach in that we wanted to observe the injected protoplast as soon as the GFP-fused protein starts to be translated within the injected protoplast. But this technique remained unsuccessful due to its complexity and the delicate nature of the protoplasts. In addition, it was found that in its present form, the microinjection set up provided by Eppendorf is not suitable for the purpose of microinjecting isolated protoplasts. For example, the injection needles are not pulled finely enough for the purpose of protoplast injection and the diameter of the mouth of the holding capillary is too large (100 µm) in comparison to the diameter of a standard mesophyll protoplast (20-40 µm).

The technique utilising the radio-labelling of proteins expressed in isolated protoplasts was then tried with OE23 and OE23-22SA constructs. When only the wild type and the mutated transit peptides fused with GFP (pOE23-GFP and pOE23-22SA-GFP) were used for these experiments, they behaved exactly like the wild type full length protein (OE23). Whereas when the full length protein with a point mutation at the site of phosphorylation (OE23-22SA) was used, an extra lower molecular weight protein band (around 5-8 kD) appeared. Since no methionines or cysteins are present in the mature part of the protein, this band would correspond to the region in the transit peptide of the OE23 protein. It is possible that this band appeared due to the slow-down in the process of import of the mutated protein. Also, as OE23 is a thylakoid protein, which is translocated into the thylakoids by the Tat pathway, it consists of two cleavable transit peptides. The stromal-directing transit peptide consists of the first 22 amino acids (2.5 kD) whereas the thylakoid-directing transit peptide comprises of amino acids from 23 to 73 (5.3 kD). Since the cleavage site predicted for the first transit peptide is near to the site of phosphorylation, it is possible that this hinders the proper cleavage of the transit peptide and its complete translocation into the stroma. In such a situation, a part of the preprotein remains stuck within the envelope translocation machineries whereas the portion which is still in the cytosol is degraded. On the other hand, it is also possible that this lower molecular weight band arises as an artifact of the labelling process.

Despite our inability to explain exactly the reason for the appearance of this band, it is of interest to note that a single mutation at the site of phosphorylation in the transit peptide of OE23 led to the occurance of an extra band after *in vivo* labelling experiments only for the full length protein but not for the pOE23-GFP chimera. This observation indicates that the mature part of a precursor protein has an influence upon its targeting and /or import behaviour. This notion is supported by the results of Pinnaduwage and Bruce (1996), who used liposomes to study the targeting properties of transit peptides in correlation with their mature forms, as well as a dual targeting study by Rudhe *et al*. (2002). In addition, regions in

mature SSU have been shown to enhance interaction with components of import apparatus (Dabney-Smith *et al*., 1999). Further studies are needed to determine if mature domain sequences contribute to the targeting activity of transit peptides in general. In addition, this brings us back to the question of the secondary structure of transit peptides. Mitochondrial presequences are characterized by their ability to form a distinct secondary structure, which is the basis for their recognition by the mitochondrial import machinery (Abe *et al*., 2000). So far no such conserved secondary structure has been identified for chloroplast targeting sequences. The deciphering of the secondary structure of the transit peptides of chloroplast-targeted proteins is likely to lead to the better understanding of the overall targeting / import process.

From among the important housekeeping genes, the NDPKs have been known since more than 60 years but there has been renewed interest in their role in cell biology and human pathology since the decreased levels of a human NDPK isoform, *nm23-H1* was found to be correlated with reduced metastasis in certain cancers (Steeg *et al*., 1988). More recently, plant NDPKs have also gained interest because of their role in signal transduction processes. In particular, the NDPK2 isoform has been shown to be involved in the phytochrome signalling pathway (Choi *et al*., 1999) and the ethylene signal transduction pathway (Novikova *et al*, 2003). It has also been reported to regulate the cellular redox state and to enhance multiple stress tolerance in transgenic plants (Moon *et al*., 2003). Its possible role in the regulation of auxin-mediated responses for plant growth and development has also been recognised (Choi *et al*., 2005).

In furthering our understanding of the various functions associated with this enzyme, the study of the NDPK2 isoform of pea and *Arabidopsis thaliana* was taken up. Whereas many studies have been conducted on *Arabidopsis thaliana* NDPK2 by different groups, to our knowledge only one has been reported for NDPK2 of pea (Luebeck and Soll, 1995). The cloning of psNDPK2 from pea cDNA, followed by its sequencing in the present study, and its import into isolated chloroplasts revealed that its primary sequence given in the database is incorrect with respect to the residue at position 77. Instead of a threonine at this position, there is an alanine. This also concurs with the NDPK2 sequences from all the other plant species investigated through alignment studies.

Two active forms of psNDPK2 were isolated by Luebeck and Soll (1995) from pea chloroplasts. Their molecular weights were 18.5 kD and a 17.4 kD, respectively. Utilising a mutagenesis approach, as discussed in detail in the Results section, we obtained two different constructs in which one cleavage site was abolished. These two proteins were then subjected to various *in vitro* and *in vivo* analyses in order to provide some insight into the reason for the occurance of the two forms of the same protein within chloroplasts. Initially it

was considered that the two forms occur due to a difference in their localisations within the chloroplast. But experimental results indicated no such difference in their localisations. They were both found to be located in the stroma and a major portion of them was associated extrinsically with the thylakoids on their stromal side. This association with thylakoids could be detached upon carbonate extraction of thylakoids. These results indicated that both active forms of the protein are localised in the stroma of chloroplasts. Further, the import behaviours of the proteins were also studied to find any difference between them, by varying the time of import, ATP concentration in the import assay as well as the age of chloroplasts. Again, no difference between the kinetics of import of the two forms could be observed. Moreover, since no change in the proportion of the two proteins was observed upon varying time scales of import, the possibility of the 18.5 kD protein being an intermediate form of the 17.4 kD mature form of the protein was ruled out.

In humans, eight NDPK isoforms have been reported. Two of which, NM23-H1 and NM23-H2, arise from two different genes and are localised in the cytoplasm of the cell. These two isoforms are reported to have arisen from gene duplication. The only difference in their functions reported till now is that NM23-H2 alone can bind to DNA whereas NM23-H1 cannot. A similar possibility was considered for the two forms of plant NDPK2 since the similarity between the sequences of plants NDPK2 and NM23-H1 and NM23-H2 is 53.9% and 51.3 %, respectively. It was considered that although the two forms of NDPK2 arose from a single gene and were localised in the same subcellular compartment, they could differ in their functions. In order to find any evidence in this regard, the functions NDPK2 could play in chloroplasts were considered. From localisation studies, it was noted that the distribution of NDPK2-GFP in chloroplasts resembles that of the distribution of nucleoids within mature chloroplasts. On further investigating the possible co-localisation of either of the two forms of NDPK2 with nucleoids, using GFP fused proteins and DAPI-staining of nucleoids, both of them co-localised with nucleoids, although this co-localisation was not absolute and a certain amount of NDPK2 proteins were also found outside the nucleoids. Furthermore, the co-localisation of APO1 (a nucleoid localised protein) with the two forms of NDPK2 further strengthened the view of their being associated with nucleoids. Interestingly, neither of the two forms of NDPK2 could be obtained when the nucleoids were isolated from chloroplasts. These results indicated towards a possible loose interaction of NDPK2 with nucleoids in contrast to a permanent tight association with them.

Furthermore, for the human isoform NM23-H2, three amino acids were found to be critical for DNA binding (Postel *et al.*, 1996). Mutations at any of these three residues led to the abolishment of the DNA-binding ability of the enzyme. Upon locating these residues in a hexamer of this protein, we found that they form a kind of a route between them, where probably the DNA binds to the NM23-H2. Two out of these three amino acids were found to

be conserved in the plant NDPK2 isoform (Zimmermann *et al.*, 1999) but not in the other isoforms, including the cytosolic NDPK1. This observation along with the likely association of NDPK2 with nucleoids hinted towards its possible binding to DNA. This was investigated by site-directed mutagenesis approach where these putative sites of DNA-binding in psNDPK2 were abolished. It was hypothesized that if the role of NDPK2 in their association with nucleoids is to bind to DNA, then their dotted distribution within the chloroplast would be lost in these mutated forms. But no such difference could be observed for both mutants. This indicated that either these positions are not the sites of DNA-binding in the chloroplast genome or that they are not critical for any binding to take place. The reason for this could be because of the differences existing between the chloroplast genome and the nuclear genome. Another possible reason for the non-observance of any difference in the distribution of wild-type and mutated forms could be the formation of mixed oligomers between the mutants and the endogenous ones and thereby providing sufficient binding sites for DNA. It is also likely that the NDPK2 remains in association with the nucleoids at all times but the DNA-binding occurs only in active nucleoids. Furthermore, upon co-transfecting protoplasts with APO1 (a nucleoid localised protein) and the mutated forms of NDPK2, no remarkable difference in their distributions could be detected. These observations led us to propose that NDPK2 may be loosely associated with nucleoids either directly or through its interaction with other nucleoid proteins. In any case, no difference between the two forms of NDPK2 could be observed either in their localisation, import behaviour and function making it likely that they have these features in common.

Another possible explanation for the appearance of the two forms of NDPK2 could be the proteolytic breakdown of the 18.5 kD form to give rise to the 17.4 kD form during the isolation of chloroplasts. If the lower band really arises due to protease activity then this is a specific proteolytic cleavage as the two bands are not only observed after *in vitro* import into isolated chloroplasts but also when the psNDPK2-GFP is transformed into isolated protoplasts obtained from pea and *Arabidopsis thaliana* leaves. The protease involved is probably not present in *E.coli* cells since the overexpression of psNDPK2 in *E.coli* gave rise to a single protein product.

The next step to promote our understanding of the function of NDPK2 within chloroplasts is to look for its interaction partners. Two approaches, among others, which can be applied to achieve this goal are:

1. Perform immuno-fluorescence analysis using antibody against NDPK2 on the one hand, and antibodies against the stromal proteins on the other. The co-localisation of the NDPK2 antibody with the stromal protein antibodies will lead to the possible indication of the interaction between the two. In this way, the requirement of the

NDPK2 can be connected with the pathways in which the stromal proteins are involved.

2. On the other hand, the conventional binding assays can be carried out in which the overexpressed soluble NDPK2 with an attached terminal tag (His/GUS) is bound to a column incubated with stroma. The proteins that bind with NDPK2 should elute with the appropriate elution buffer and can be sequenced.

The exact localisation of NDPK2 within plants has been a matter of dispute as for *Arabidopsis thaliana* it was shown to be in the cytosol and the nucleus, whereas it was reported to be in chloroplasts in pea, spinach and *Brassica campestris*. We present here various lines of evidence to show that the NDPK2 of *Arabidopsis thaliana* is present in chloroplasts. Targeting of the *in vitro* translation product of *Arabidopsis thaliana* atNDPK2 into isolated chloroplasts indicated its organellar localisation. The protein has a predicted targeting peptide at its N-terminus which is removed upon import into the chloroplast. atNDPK2-GFP fusion protein, when transformed into isolated protoplasts, was expressed in chloroplasts whereas the protein with a deletion of the transit peptide was seen to be in the cytosol. These results clearly indicate that the N-terminal extension of atNDPK2 represents the targeting signal to chloroplasts. The GFP fusion results presented in the present study are in direct contradiction to the earlier published results of Choi *et al*. (1999) and Zimmermann *et al*. (1999) where they showed the atNDPK-GFP fusion protein to be in the cytosol and the nucleus. The reason for the difference observed between their and our results is likely to be due to the fact that Choi *et al*. (1999) fused the GFP to the atNDPK2-Δ1-79 sequence, which lacked the transit peptide, whereas Zimmermann and co-workers had fused the full length of atNDPK2 and the construct without the transit peptide (atNDPK2-Δ1-79) at the C-terminus of the GFP. In these cases, the role played by the transit peptide in the translocation of the protein into chloroplasts, was circumvented by its complete absence from the sequence or by the presence of the GFP protein before it. It is known, through our experiments and literature (Chiu et al., 1996) that GFP in itself accumulates in the nucleus of the transfected cell. Therefore, these studies used wrong constructs for establishing the location of NDPK2 within the *Arabidopsis thaliana*.

Further, Zimmermann *et al*. (1999) listed three points to argue the unlikelihood of the presence of NDPK2 within chloroplasts. These points are listed (in italics) and discussed below:

1. *There is no homologous pattern of its* (NDPK2) *N terminus with chloroplast leader sequences from other plants.*

At the primary sequence level, chloroplast transit peptides are highly divergent in length, composition and organisation and there is little conservation between them. Although some general features have been identified for them e.g. they have few acidic residues and are rich in hydroxylated residues, there is no known motif or obvious sequence pattern that identifies proteins as chloroplast targeted. The exact nature of the 'biological' signal that the cell interprets is not yet known (Schein et al., 2001).

2. *The atNDPK2-GFP fusion and the leader-free deletion (Δ1-79) are localized in the cytosol and nucleus but not in Arabidopsis chloroplasts.*

As mentioned above, due to the fusion of the GFP at the N-terminus of atNDPK2 sequence, the transit peptide could not exert its function in the translocation of the protein. Our experiments proved that with GFP fusions at the C-terminus of atNDPK2 the protein was transported into chloroplasts whereas the atNDPK2-Δ1-79-GFP protein remained in the cytosol.

3. *The expressed protein is not transported into isolated chloroplasts in vitro (data not shown).*

In the present study, upon the incubation of the *in vitro* translated atNDPK2 with isolated chloroplasts in an import assay, this protein was imported into chloroplasts and it gave rise to two imported products, similar to that observed for pea in an earlier study (Luebeck and Soll, 1995). The localisation of these proteins within chloroplasts could be affirmed by post-import thermolysin treatment of chloroplasts.

RT-PCR and RACE-PCR experiments conducted to investigate the possibility of the occurrence of alternative splicing for the atNDPK2 also did not reveal any truncated form of the protein which could remain in the cytosol. This further strengthened the view that the NDPK2 protein of *Arabidopsis thaliana* is localised within chloroplasts, like its homologues from other plant species. Further, a very high sequence similarity was found between the *Arabidopsis thaliana* NDPK2 and NDPK2 from pea, spinach and *Brassica rapa*. It was 94% with *Brassica rapa*, 85.5% with spinach and 81.3% with pea NDPK2. This high similarity between the sequences suggests that they share not only similar function, but also the same subcellular localisation and import pathways. This is further supported by phylogenetic analysis where the NDPK2 of *Arabidopsis thaliana*, pea, spinach, *Nicotiana tabacum* and *Brassica campestris* group together in one branch of the phylogenetic tree (Dorion *et al*., 2006).

In the study conducted by Choi *et al*. (1999) an association between atNDPK2 and phytochromes was determined by the yeast two-hybrid screening method. In another report

(Im *et al*., 2004), the authors applied *in vitro* binding assays to show that the C-terminal signal-transduction domain of phytochrome is critical for its interaction with NDPK2 protein. Since our experiments clearly show atNDPK2 to be present in chloroplasts and not in the cytosol, we believe that the *in vitro* binding assays in the above studies are unspecific binding as the C-terminus of phytochromes contain a histidine kinase-related domain (Kim *et al*., 2002) which could easily interact with NDPK2 in a highly unspecific manner. Choi *et al*. (1999) had also analysed *ndpk2* mutant plants and found that NDPK2 is involved in the phytochrome A mediated far-red high-irradiance response in *Arabidopsis thaliana*. Although this may be true it does not imply the presence of NDPK2 within the same cellular compartment as phytochromes. In the present work, we transformed *Arabidopsis thaliana* protoplasts with atNDPK2-GFP under different light conditions. Under no condition it was found to be present in the cytosol or the nucleus. On the other hand, similar studies conducted with phytochrome-A-GFP fusion proteins could show its light-induced nuclear import from the cytosol (Kircher *et al*., 1999; Kim *et al*., 2000). Moreover, although the phytochromes were speculated to be present in chloroplasts for a long time, the present evidences indicate that this is not the case and it is widely accepted now that they are present in the cytosol and nucleus (Yamaguchi *et al*., 1999). From all these results taken together, we conclude that the atNDPK2 is exclusively localised within chloroplasts and may be involved in the various environmentally induced signalling pathways.

Taken together, regarding the function and import of the two isoforms of NDPK2 in pea, no differences between the two could be determined. However, it could be clearly demonstrated that both the forms of psNDPK2 are present in the stroma and are associated with thylakoids and nucleoids in chloroplasts. Pertaining to the atNDPK2, it could be shown conclusively that it is exclusively localised in chloroplasts like all its counterparts in the higher plants studied till date. Though this raises new questions concerning the exact function of atNDPK2 in the regulatory pathways where it has been shown to play a role, the dispute regarding its localisation could be settled conclusively.

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11. Ehrenwörtliche Versicherung

Die vorliegende Dissertation wurde von Dipl. Biol. Rita Sharma selbständig und ohne unerlaubte Hilfe angefertigt. Die Autorin hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

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