

Thesis for the Master's degree in Molecular Biosciences

Main field of study in Molecular Biology

**Expression of a histidine-tagged RNA
pyrophosphohydrolase in *Chlamydomonas reinhardtii* for
localization studies**

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60 study points

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Summary

RNA pyrophosphohydrolase (RppH) catalyzes the removal of pyrophosphate from 5' triphosphorylated RNAs thereby initiating RNA degradation. The enzyme has originally been identified in bacteria but homologs are present in eukaryotes where they are thought to be located in plastids or mitochondria. A homolog of the bacterial RNA pyrophosphohydrolase is present in the unicellular green alga *Chlamydomonas reinhardtii* suggesting that *Chlamydomonas* RppH has a role in mRNA degradation in the chloroplast of the alga. The purpose of this project was to determine the localization of the RppH homologue in *C. reinhardtii*. Localization was investigated using two different constructs, a histidine-tagged version of the *Chlamydomonas rppH* and a histidine-tagged *5'rppH-GFP* construct.

A plasmid vector containing *Chlamydomonas rppH*-6xHN was introduced into *C. reinhardtii* by nuclear transformation. PCR, RT-PCR, sequencing, and DNA and RNA blotting techniques were used to identify positive transformants at the DNA and RNA level. In addition, transformants carrying a histidine-tagged *5'rppH-GFP* construct, that has previously been transformed and verified to be present at the DNA level, was investigated by RNA blotting. SDS-PAGE, antibodies, mass spectrometry and chloroplast isolation were used to evaluate protein expression from both constructs. In addition a protein activity assay was developed in order to confirm that the *Chlamydomonas* RppH homolog has RNA pyrophosphohydrolase activity.

Accumulation of RppH-6xHN and 5'RppH-GFP-6xHN proteins in transformants has been detected but the results need to be further substantiated. Localization of RppH was not possible in the time frame of the project because of problems with antibody specificity and with the chloroplast isolation procedure. Further work should focus on analyzing additional transformants and on localization of 5'RppH-GFP-6xHN using confocal microscopy.

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1 INTRODUCTION

1.1 The Nudix family and RppH

1.1.2 The Nudix family

The Nudix family is a protein family of phosphohydrolases. Using water-mediated catalysis Nudix hydrolases break a phosphate bond in a wide range of organic pyrophosphates with varying degrees of substrate specificity. The number of Nudix genes varies widely from 0 to over 30 in prokaryotes and simple eukaryotes, and reflects the organism's metabolic complexity and adaptability.

The family consists mainly of pyrophosphohydrolases that act upon substrates with a general structure of nucleoside diphosphate linked to another moiety, **X** (NDP-X), thereof the name Nudix. The enzymes hydrolyse the substrates yielding NMP and P-X (McLennan 2006).

There are two components to the Nudix family, the Nudix fold and the Nudix motif. The sequence motif G-x(5)-E-x(5)-[UA]-x-R-E-x(2)-E-E-x-G-U also called the Nudix box (PROSITE PS00893) is the catalytic site and forms a loop- α -helix-loop structural motif (McLennan 2006). The Nudix box also contains metal binding amino acids and is part of the second component, the Nudix fold. The Nudix fold is a $\alpha/\beta/\alpha$ sandwich which together with the Nudix box contains additional regions. These regions determine enzyme specificity and mechanism and therefore differ in the various enzymes. The enzymes have different requirements concerning number of divalent cation ions and perform the substitution at various positions in the substrates (McLennan 2006). Members of the GDP-mannose hydrolase sub-family even catalyse substitution by water at carbon, instead of phosphorus, which is what most of the enzymes in the family do (Legler et al. 2002).

Nudix enzymes perform important roles in metabolism, not only because they have a regulatory role, but also because they protect the cells against damaged nucleoside triphosphates. Their substrates also include intact nucleoside triphosphates, capped RNA, dinucleotide coenzymes, nucleotide sugars, and dinucleoside polyphosphates (McLennan 2006).

1.1.3 The role of RNA pyrophosphohydrolase in mRNA degradation

RNA pyrophosphohydrolase is a member of the Nudix family and an enzyme that probably has an important role considering RNA degradation. It is now believed that RppH converts RNA to a monophosphorylated stage by removing pyrophosphate from the 5' end. While eukaryotes use a 5' cap structure to protect their mRNA from degradation, prokaryotes do not modify the 5' end of their mRNA further. Studies indicate that the 5' triphosphates on mRNAs in prokaryotes have a similar function as the cap structure in eukaryotes, protecting the mRNA from degradation (Celesnik et al. 2007). Celesnik et al. (2007) further discovered that RNA decay can be triggered by converting the 5' terminus from a triphosphate to a monophosphate. 5' monophosphorylated mRNAs are better substrates for the endonuclease RNase E, and therefore this non-nucleolytic event marks transcripts for degradation.

RppH, previously named NuH/YgdP, was identified in *E. coli* as an RNA pyrophosphohydrolase in 2008 (Deana et al. 2008). Deana et al. (2008) showed that RppH removes pyrophosphate from the 5' end of a triphosphorylated RNA *in vitro*, and that the enzyme by converting RNA to a more labile monophosphorylated stage accelerates degradation of hundreds of *E. coli* transcripts *in vivo*. This changed the long-standing paradigm that mRNA degradation in *E. coli* is initiated with endonucleolytic cleavage by RNase E (Bail and Kiledjian 2009). After generation of a 5' monophosphorylated mRNA by RppH, the RNA is further degraded. In *Bacillus subtilis* two different pathways are possible. In the first pathway, removal of the diphosphate facilitates endonucleolytic cleavage of the mRNA by RNase E (Condon 2007). The fragments created by RNase E are then degraded by 3' to 5' exonucleases. In the second pathway, the 5' monophosphorylated mRNA is subject to 5' to 3' exoribonuclease degradation (Condon 2007).

1.2 *C. reinhardtii*

1.2.1 *C. reinhardtii* as a model organism

Chlamydomonas reinhardtii, hereafter referred to as *C. reinhardtii*, is one of several species in the genus *Chlamydomonas*. The genus consists of unicellular algae containing a chloroplast, one or several pyrenoids, a distinct cell wall, and two anterior flagella (Figure 1.1) (Harris 2001). *C. reinhardtii* was isolated from soil habitats in North America during the 1940s and 1950s, and is widely distributed around the world in soil and fresh water. Morphologically *C. reinhardtii* is an oval shaped cell enclosed by a seven-layered cell wall, primarily built up by hydroxyproline-rich glycoproteins. Furthermore the organism contains a single chloroplast, occupying approximately two thirds of the cell, endoplasmic reticulum, Golgi apparatus, mitochondria, contractile vacuoles, nucleus and other structures (Figure 1.1) (Harris 2001).

Several species within the genus *Chlamydomonas* have been used in research, but *C. reinhardtii* is by far the most popular (Harris 2001). The advantages in using *C. reinhardtii* as a model organism are many. For instance, the alga is nutritionally simple, can reproduce both asexually and sexually and has short generation time. Furthermore *C. reinhardtii* is a haploid and can be handled by standard microbial techniques, despite being a eukaryote. These and other advantageous properties, like available genetic information and motility, makes the species ideal as model system (Harris 2001).

The main research areas using the model system of *C. reinhardtii* includes flagellar function and structure, basal body (centriole), genetics, generating biofuels, chloroplast biogenesis, cell-cell recognition and cell-cycle control (Harris 2001). Due to several attributes, *C. reinhardtii* has also been proven especially useful in photosynthetic research. The alga is both a heterotroph and a facultative autotroph, meaning it can be grown photosynthetically, heterotrophically and mixotrophically (Heifetz et al. 2000). By growing *C. reinhardtii* nonphotosynthetically, using acetate as carbon source, wild-type strains can grow in the dark and photosynthetic mutants become viable (Harris 2001). Furthermore the sequence of the *C. reinhardtii* genome is available. In the late 1980s a chloroplast transformation system based on microprojectile bombardment was developed, allowing for genetic modifications of the chloroplast genome by homologous recombination (Boynton et al. 1988, Blowers et al. 1989). In fact, successful transformation of all the three genomes, nuclear, plastid and mitochondrial has been reported (Harris 2001).

Another favorable feature of *C. reinhardtii*, which is of particular interest in this project, is the ability to detect nuclear genes that regulate gene expression in the chloroplast (Harris 2001). An example is the chloroplast encoded *psaA* gene. This gene assembles by a trans splicing process that has been shown to be affected by at least 14 nuclear encoded genes (Harris 2001).

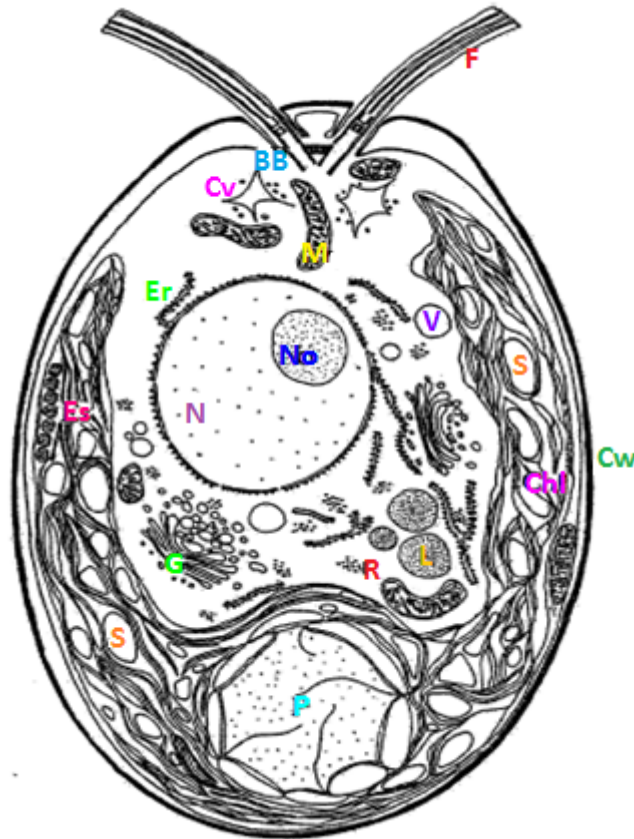


Figure 1.1 An overview of the content of the *C. reinhardtii* cell. BB; basal bodies, Chl; chloroplast, Cv; contractile vacuole, Cw; cell wall, Er; endoplasmatic reticulum, Es; eyespot, F; flagella, G; golgi apparatus, L; lipid body, M; mitochondria, N; nucleus, No; nucleolus, P; pyrenoid, R; ribosomes, S; starch grain, V; vacuole. Modified from (Harris 2001).

1.2.2 Vegetative and sexual life cycles of *C. reinhardtii*

The wild-type of *C. reinhardtii* is relatively easy to grow. The alga can either be grown in defined liquid or on agar media at neutral pH, without any supplementary vitamins or co-factors (Harris 2001). Depending on the growth conditions, a typical growth rate will give a tenfold increase in cell number each day. Optimal temperature is from 20°C to 36°C. Under ideal growth conditions the alga reproduces only through mitosis, but sexual propagation can be triggered by nitrogen starving the cells. There are two genetically determined mating types, mt(+) and mt(-). When mixed, + and - gametes form mating pairs that fuse and form diploid cells that become heavy-walled zygospores. The zygospore also functions as a dormant form of the species in the soil (Harris 2001). Under favorable conditions meiosis occurs and the zygospore releases four flagellated haploid cells; two of

the + and two of the – mating type (Proschold et al. 2005). Alternating periods of light and dark can be provided to synchronize cell division (Lien and Knutsen 1979).

1.2.3 The nuclear genome of *C. reinhardtii*

Because of its extensive metabolic flexibility, *C. reinhardtii* is a highly adaptable species with the ability to survive variations in nutrient availability and grow in different environmental niches (Grossman et al. 2007). In 2007 the approximately 120-megabase nuclear genome sequence of *C. reinhardtii* was published (Merchant et al. 2007). The sequence has a GC-content of approximately 64%, which is relatively high and in some cases produces difficulties in cloning. Merchant et al. (2007) reported that the *C. reinhardtii* genome consists of 17 linkage groups, presumably corresponding to 17 chromosomes, confirming the results received from electron microscopy (Storms and Hastings 1977). Considering the distribution of genes, simple sequence repeats, and transposable elements, the density is almost uniform in the genome (Merchant et al. 2007). In addition, the ribosomal RNA genes in the *C. reinhardtii* genome are placed in tandem arrays, which are the case for most eukaryotes (Merchant et al. 2007).

1.2.4 Nuclear transformation

Transformation of the *C. reinhardtii* nuclear genome results mainly in heterologous recombination. It has been performed using several methods, including glass bead agitation (Kindle 1990), silicon fiber agitation (Dunahay 1992), electroporation (Brown et al. 1991), and particle bombardment (Debuchy et al. 1989, Kindle et al. 1989). There are several advantages using glass bead agitation. First of all no specialized equipment is required and the method is in addition both inexpensive and simple. By agitating cell wall-less mutants with glass beads, DNA, and polyethylene glycol a transformation rate of 10^3 transformants per μg plasmid DNA has been reported (Kindle 1990).

To make nuclear transformation more efficient wall-deficient mutants are widely used. Mutants with altered cell walls were isolated in the early 1970s by David and colleagues, who divided the mutants into three classes, A, B, and C (Davies and Plaskitt 1971, Hyams and Davies 1972, Davies and Lyall 1973). Studies have shown that most of these mutants produce normal amounts and set of cell wall polypeptides, but are unable to assemble them into complete walls (Voigt et al. 1997). The most used wall-deficient mutant is cell wall-less mutant 15 (cw15), which is placed in class C (Davies and Plaskitt 1971). Wild type cells contain, as mentioned, a seven layered cell wall (Harris 2009). Structures resembling the outermost layer of the wild type wall is found in cw15, however the mutant fails in assembling the central layers, W2-W6 (Monk et al. 1983).

1.3 Chloroplasts

1.3.1 Protein import into the chloroplasts

All plastids evolved from undifferentiated semiautonomous proplastids (Soll et al. 2002). They contain their own genome, but most of the chloroplast proteins are coded for in the nuclear genome and synthesized in the cytosol of the cell. Thus, import of proteins into the chloroplast from the cytosol is an important process. This transport has been shown to require ATP (Grossman et al. 1980). Once inside the organelle, the polypeptides are targeted to specific chloroplast compartments, and assembled into their functional state. Targeting of newly synthesized proteins to specific organelles and compartments within them is known to involve three general mechanisms: posttranslational, cotranslational and mRNA-based mechanisms (Uniacke and Zerges 2009).

A few thousand proteins are imported into the chloroplasts of plants and algae. These proteins are believed to be synthesized at random locations in the cytoplasm before they are imported post-translationally (Chua and Schmidt 1979, Carde et al. 1982). In a posttranslational mechanism the proteins are selected to the chloroplast by the import machinery, if they contain a transit peptide (Soll et al. 2002). Additionally a few proteins are trafficked to chloroplasts through the secretory system.

Both the outer membrane and the inner membrane of the chloroplast contain an import apparatus, called Toc (translocon at the outer membrane) and Tic (translocon at the inner membrane) (Soll et al. 2002). Together with cytosolic factors, these complexes target the cytosolic proteins to the chloroplast surface, before they import the proteins to the stroma of the chloroplast. Inside the stroma, the N-terminal signal sequence of the protein is cleaved off, new signals are revealed and the protein is further sent to its final destination (Soll et al. 2002).

Several proteins translocated to the stroma, undergo further targeting to either reach the thylakoid membrane or the thylakoid lumen. Together at least four pathways, two to the thylakoid membrane and two to the thylakoid lumen have been discovered (Soll et al. 2002). To enter the thylakoid lumen the proteins have to cross all three chloroplast membranes either by using a Sec-related pathway (Tat-system) or a Sec-independent pathway (Δ pH-dependent pathway). The pathways relocating proteins into the thylakoid membrane are named the SRP/FtsY-dependent pathway and the SRP-independent pathway (Dalbey and Robinson 1999).

1.3.2 Transcription in the chloroplast of *C. reinhardtii*

There are around 100 genes in the *C. reinhardtii* chloroplast genome. They are organized and transcribed in two different ways, either singly or as part of a multi-gene transcription unit (Klein 2009). The transcription machinery in *C. reinhardtii* chloroplasts is prokaryote-like with respect to promoter sequences and RNA polymerase subunits. In gene content the genome has great resemblance to land plants, however this is not the case for gene order, where the land plant model and *C. reinhardtii* model have several differences (Klein 2009). In higher land plant chloroplasts the genes are primarily organized in operons, while many genes in the *C. reinhardtii* chloroplast seem to be transcribed from their own promoters (Sugiura 1992).

In plastids of flowering plants we find two types of RNA polymerase, one which is encoded in the nucleus (NEP) and one which is encoded in the plastid (PEP) (Klein 2009). *C. reinhardtii* chloroplasts however, seem to contain only one of the DNA dependent RNA polymerases, the PEP (Smith and Purton 2002). The PEP RNA polymerase is homologous to typical eubacterial RNA polymerases (Klein 2009).

Chloroplast transcription in *C. reinhardtii* is regulated by growth of the cells in a 12-hour light/12-hour dark cycle (Leu et al. 1990, Salvador et al. 1993, Hwang et al. 1996). Normally the transcript level is at its lowest at the beginning of the dark period and highest in the start of the light period, and these fluctuations are resulting from fluctuations both in RNA degradation and transcription (Hwang et al. 1996). A study performed by Hwang et al. (1996) concluded that transcription of several chloroplast genes in *C. reinhardtii* is controlled by a circadian clock, whereas light controls RNA degradation. Like in bacteria, negatively supercoiled DNA is transcribed much more efficiently than relaxed DNA (McClure 1985).

1.3.3 Processing of chloroplast mRNA and mRNA stability in *C. reinhardtii*

There are a number of processes that control chloroplast gene expression including transcription, post-transcriptional processing, translation, and post-translational modifications (del Campo 2009). Messenger RNA processing and degradation in chloroplasts are in several ways similar to the corresponding processes in bacteria. Intron splicing, internal cleavage of polycistronic RNAs, RNA editing (not observed in *C. reinhardtii*), and endonucleolytic or exonucleolytic cleavages are some of the ways transcripts can be processed (Monde et al. 2000). However, since nearly all of the *C. reinhardtii* chloroplast genes seem to be transcribed as monocistronic RNAs, processing of transcripts might be less important for gene expression than translation (Rochaix et al. 1989, Zerges and Rochaix 1994, del Campo 2009).

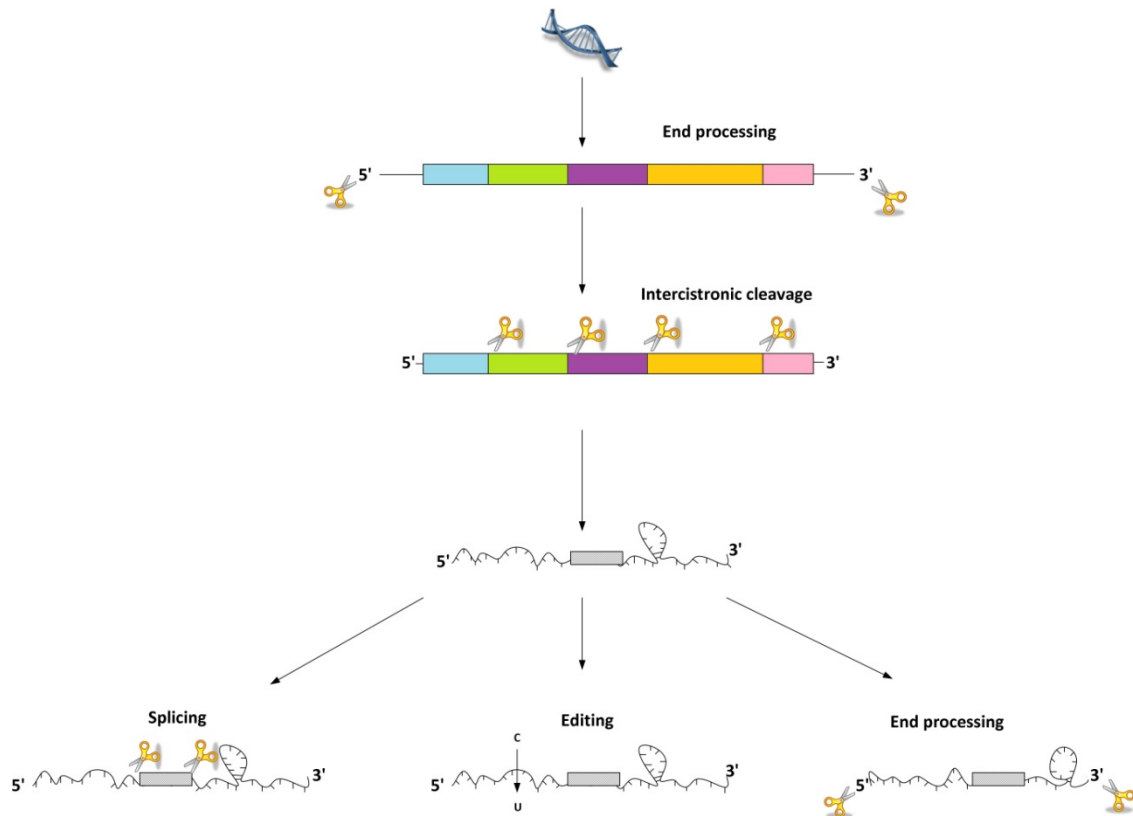


Figure 1.2 Processing/maturation of mRNA in chloroplasts. In chloroplasts a gene cluster is transcribed from a single promoter, followed by end processing and intercistronic cleavage. However, in *C. reinhardtii* genes are usually transcribed as monocistronic RNAs, and intercistronic cleavage is therefore less important. Monocistronic RNAs are further modified by splicing, editing and end processing. Editing is not observed in *C. reinhardtii*. It is believed that the 5' ends of *C. reinhardtii* chloroplast RNAs are processed because they lack three terminal phosphate groups (Herrin 2009). Based on (Stern et al. 2010).

The stability of the transcripts in chloroplasts is mainly influenced by the sequences in the 5' untranslated and 3' untranslated regions. If deleted or mutated a reduction in transcript accumulation and translation is observed (Anthonisen et al. 2001, Salvador et al. 2004, Suay et al. 2005). Evidence indicates that the sequences in 5' and 3' UTRs fold into specific secondary structures (Higgs et al. 1999, Anthonisen et al. 2001, Zicker et al. 2007). It is believed that nucleus encoded proteins known to be required for transcript stability bind to these cis-acting stabilizing elements (Herrin 2009). Mutants known to lack specific transcripts in the chloroplast have been shown to be deficient in proteins binding to the 5'UTR. This suggests a more variable and specific protection of the sequences in the 5'UTR than of the 3'UTR in *C. reinhardtii* chloroplasts (Salvador et al. 2011).

Chloroplast mRNA in *C. reinhardtii* may be processed at their 5' ends, due to reports of failure in capping the mRNA with a 5' trimethylguanosine cap *in vitro* (Herrin 2009). For capping mRNAs, three phosphate groups at the 5' end of the mRNA are needed. Lack of capping in such an experiment therefore implies that the mRNA 5' end is processed. A function for this type of processing is for most mRNAs not clear. However, it is suggested that a function may be to create better RNA products for translation (Bruick and Mayfield 1998) and that processing could create more stable mRNAs (Nickelsen et al. 1999).

The 3' end of mRNAs in chloroplasts is determined by post-transcriptional processing. Studies on the *atpB* gene of *C. reinhardtii* indicate that the 3' end of the transcripts is processed in two steps. First endonucleolytic cleavage is performed 10 bp downstream of the inverted repeat (IR), a stem loop that forms at the 3' end. This is called the endonucleolytic cleavage site (ECS). The second step is 3' to 5' digestion with exonucleases, which stops at the IR terminus (Stern and Kindle 1993). The IR stemloop has been shown to stabilize the *atpB* mRNA (Stern et al. 1991). In general exonucleases, endonucleases, and RNA-binding proteins all participate in 3' end maturation of mRNAs. In *C. reinhardtii* there is evidence pointing toward preferred translation of mRNA with the correct 3' ends (Rott et al. 1998) and that it also can function in providing 3' to 5' exonuclease resistant termini (Drager et al. 1996). The role of the 3'UTR in the chloroplast of *C. reinhardtii* thus differs from the 3'UTR in bacteria, being more involved in transcript stability and preventing 3' to 5' exonucleolytic degradation, while the bacterial 3'UTR mostly is involved in transcription termination (Bollenbach et al. 2004).

The half-life of chloroplast mRNA in *C. reinhardtii* is regulated by nutrient availability, plastid development, and as mentioned in chapter 1.3.2 light (Herrin 2009). However the mechanisms underlying these controls are poorly understood. Several nuclear-encoded proteins that influence processing and stabilization of chloroplast transcripts have been characterized. An example are proteins that bind to transcripts of the chloroplast *petA* gene in *C. reinhardtii*, which encodes cytochrome f (del Campo 2009). Stability of *petA* transcripts depends on the nucleus encoded factors MCA1 and TCA1. MCA1 is required for stable accumulation of *petA* transcripts, while TCA1 is needed for translation (Raynaud et al. 2007).

1.3.4 Degradation of chloroplast mRNA

All RNAs are subject to degradation which enables cells to remove damaged RNAs, recycle nucleotides, and dispose of processing byproducts. Degradation is together with processing and synthesis controlling accumulation of RNAs (Herrin 2009), and knowledge of mRNA decay is essential for understanding overall regulation of gene expression and mRNA turnover. In general ribosomal RNAs and transfer RNAs have longer half-lives compared to messenger RNAs (Herrin 2009).

Messenger RNA degradation in chloroplasts is usually quite efficient, and seldom accompanied by high level accumulation of intermediates (Herrin 2009). Considering these findings, it is therefore believed that the initial mRNA degradation step is the important rate-limiting step. For chloroplast protein-coding mRNAs the initial degrading step is unknown (Herrin 2009).

As mentioned transcripts of the *atpB* gene were shown to become destabilized by deletion of the 3'IR (Stern et al. 1991). Several studies argue for a 3' to 5' exonuclease degradation activity in the chloroplast of *C. reinhardtii*. For instance it has been shown that replacing the 3'IR of *atpB* transcripts with a poly(G) sequence, results in accumulation of a discrete *atpB* transcript terminating at the poly(G) sequence (Drager et al. 1996). Another mechanism which is believed to take place in prokaryotes and plastids is a polyadenylation-dependent degradation mechanism. The pathway starts with endonucleolytic cleavage, continues with addition of a poly(A)-rich tail and ends with exonucleolytic degradation (Slomovic et al. 2006). In general mRNA decay in plastids is believed to be

performed by homologues of the bacterial endoribonucleases RNase E/G and RNase J type in addition to the two 3' to 5' exoribonucleases RNase II and PNPase (Mudd et al. 2008, Schein et al. 2008, Zimmer et al. 2009, Stern et al. 2010). RNaseJ, originally reported as an endoribonuclease, has also been found to possess 5' to 3' exonuclease activity (Mathy et al. 2007).

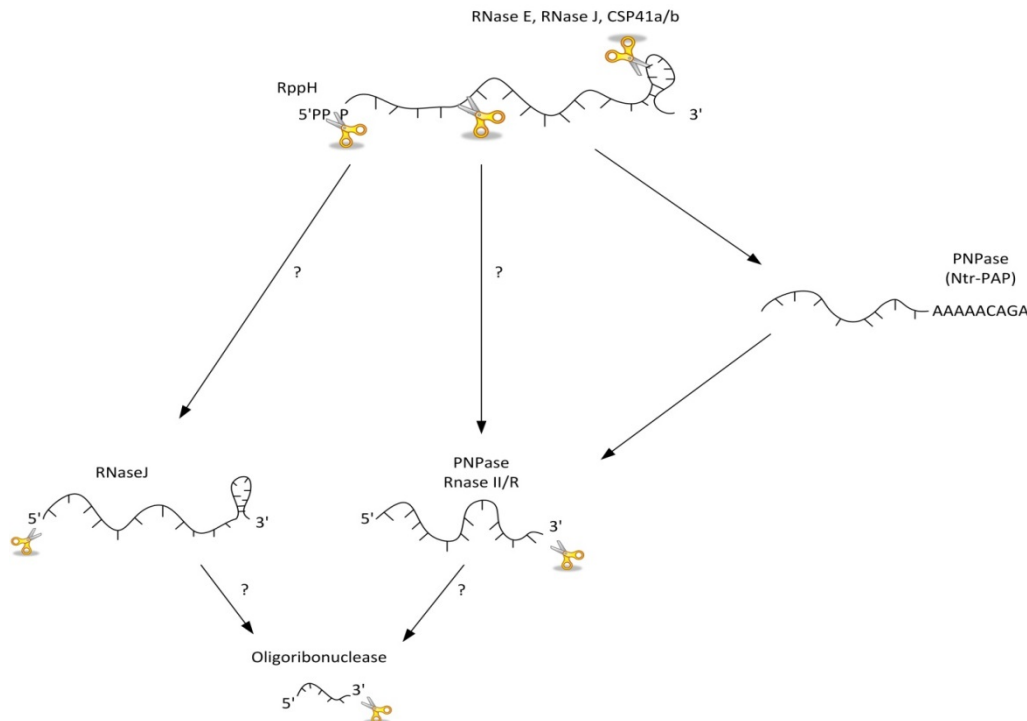


Figure 1.3 Degradation of mRNA in chloroplasts. The pathway to the right is the polyadenylation-stimulated RNA degradation pathway. It starts with endonucleolytic cleavage, which is believed to be performed by a homologue of the *E. coli* enzyme RNase E. In *E. coli* it has been shown that RNase E cleavage is favored when 5' end pyrophosphate is removed by an RNA pyrophosphohydrolase (RppH). Another endonuclease present in the chloroplast is CSP41, which may be involved in initial cleavage. In chloroplasts polyadenylation is performed by the bifunctional PNPase, which produces heteropolymeric poly(A)-rich tails, and perhaps Ntr-PAP, which produces homopolymeric poly(A) tails. The third step, 3' to 5' exonucleolytic degradation, is performed by PNPase and RNase II/R. Question marks show possible pathways, which have not yet been proven. 5' to 3' exonucleolytic degradation is predicted to be performed by RNase J in the organisms harboring the enzyme. After 3' to 5' and 5' to 3' exonucleolytic degradation, the residual oligomers might be degraded by oligoribonuclease, as shown in *E. coli* (Deana et al. 2008). Based on (Schuster and Stern 2009).

Degradation of RNA by 5' to 3' exonucleases is neither observed in bacteria nor in plants. However, in *C. reinhardtii* chloroplasts evidence for mRNA degradation beginning at the 5' end within the 5'UTR has been reported (Zicker et al. 2007). Further studies suggest that these 5' to 3' mRNA depending pathways follow sequence- and condition-dependent modes in chloroplasts (Salvador et al. 2011). The pathways involve 5' to 3' exoribonucleolytic and, most likely, endoribonucleolytic activities. Evidence also indicates that there is a 5' to 3' processive endonuclease, probably similar to RNase E, that degrades RNA downstream of the 3' processive signals (Hicks et al. 2002). A homologue of RNase E in *C. reinhardtii* has not been found. However, an *E. coli* RNase J homologue, that is capable of degrading RNA 5' to 3', appears to be present (Herrin 2009).

1.4 RppH in *C. reinhardtii*

RppH was, as mentioned, discovered in the prokaryotic organism *E. coli*. Since chloroplasts descend from free living bacteria and contain prokaryotic features, species that harbor a chloroplast, as *C. reinhardtii*, might inhabit an RppH homologue. Additionally previous studies showed that chloroplast mRNAs in *C. reinhardtii* could not be capped *in vitro* (Herrin 2009), implying that they lack triphosphates at their 5'ends. The reason was thought to be 5'end processing. However with the new findings of a pyrophosphohydrolase in *E. coli* another possibility for the lack of triphosphates at the 5' terminus of chloroplast mRNA can be considered, being a result of pyrophosphate removal by an RppH homologue.

BLAST searches with the newly discovered *rppH* sequence from *E. coli* were performed (Uwe Klein and Maria L. Salvador), and several Nudix family genes were found in *C. reinhardtii*. One of the genes, which had most homology to the *E. coli rppH* gene, was chosen for further studies. This *rppH* homologue is located in the nucleus of *C. reinhardtii* and its transcript translated on cytosolic ribosomes, but the protein is thought to function in the chloroplast of the alga. A critical point of the hypothesis is therefore to show that the *Chlamydomonas* RppH protein is located in the chloroplast.

2 AIMS OF STUDY

The main goals of the project:

- To make a histidine-tagged version of the *Chlamydomonas* RppH protein
- To express the histidine-tagged RppH in *C. reinhardtii* cells in order to localize the protein.

The main goals were further divided in several sub-goals:

- Cloning of the *Chlamydomonas rppH* gene, coding for a histidine-tagged version of the protein, into a transformation vector.
- Insertion of the *Chlamydomonas rppH* gene into the nuclear genome of *C. reinhardtii*.
- Selection and screening of *C. reinhardtii* transformants by analyzing positive transformants at the DNA and RNA level.
- Expressing a histidine-tagged version of the *Chlamydomonas* RppH in *E. coli*.
- Investigate the specificity and function of histidine-tag antibodies.
- Localize RppH in *C. reinhardtii* by different strategies as immunodetection using histidine-tag antibodies and confocal microscopy, chloroplast isolation, SDS-PAGE, and mass spectrometry.

3 MATERIALS AND METHODS

3.1 Work with DNA

3.1.1 Agarose gel electrophoresis

A 1% agarose gel containing ethidium bromide placed in a tray with TAE buffer [1X], was used in all experiments. The gel was prepared as follows:

Procedure:

- Mix 60mL TAE 1X buffer and 0.6g of agarose. Heat until boiling in a microwave.
- Cool the solution down to 50°C and add 10µL of ethidium bromide [1mg/mL].
- Pour the solution into a tray, insert a comb, and wait for solidification.

1 µL of gel loading buffer was added to 9µL of sample, and 10µL was loaded on the gel. The samples were fractionated for 30 minutes by applying a voltage of 90V. For estimation of size and concentration of DNA-samples, a 1kB plus ladder (Invitrogen) was used.

3.1.2 Purification of DNA fragments from agarose gel

Agarose gel electrophoresis was performed on the DNA-samples to separate the DNA fragments as explained in section 3.1.1. After electrophoresis a gel slice containing the DNA fragment of interest was cut out. The DNA was purified from the gel by using an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

Procedure:

- Cut out a agarose band containing the DNA fragment of interest and place the gel slice in a 1.5mL microcentrifuge tube. Weigh and calculate the weight of the agarose gel slice.
- Add 10µL Capture buffer type 3 for each 10mg of gel slice. Incubate at 60°C until the agarose is completely dissolved. Mix by inversion occasionally.
- Transfer up to 800µL of the sample mixture onto an assembled GFX Microspin column and Collection tube, and incubate in room temperature for 1 minute. Centrifuge at 16000g for 30 seconds.
- Discard the flow through, and repeat the previous step until the entire sample is loaded.
- Add 500µL Wash buffer type 1 to the GFX Microspin column.
- Spin the assembled GFX Microspin column and Collection tube at 16000g for 30 seconds.
- Discard Collection tube and transfer the GFX microspin column to a new 1.5mL microcentrifuge tube.
- Add 10-50µL Elution buffer type 4 or type 6 to the center of the GFX microspin column and incubate for 1 minute. Spin at 16000g for 1 minute to recover the DNA. The DNA can be stored at -20°C.

3.1.3 DNA quantification

Dot spot analysis and spectrometry measurements at $O.D_{260nm}$ were used to estimate DNA concentrations. Dot spot analysis was performed on samples before ligation, while spectrometry measurements were done to quantify the DNA after maxi prep. In addition some samples used in ligation were also quantified with nano drop.

Dot spot

- A set of standards with DNA in water solutions were prepared.
 - Mix 2 μ L of each of the standards and 2 μ L of each of your samples with 2 μ L of ethidium bromide [2 μ g/mL] in drops on a plastic petri dish which is transparent to UV light.
 - Compare the sample fluorescence with the fluorescence from the standards under UV light to estimate DNA concentration.
- Usually the samples need to be diluted to match the range of the standards.

Spectrometry measurements

Measure $O.D_{260nm}$ for a diluted sample. $O.D\ 1.0 = 50\mu\text{g DNA/mL}$

3.1.4 Polymerase chain reaction (PCR)

The polymerase chain reactions were performed with a Taq polymerase (Sigma-aldrich) on a T1 Thermocycler (Biometra) machine. Primers used are listed in Appendix III.

Subcloning

In the subcloning three different PCR reactions were preformed, all by using the following settings:

- Step 1: 94°C, 1 minute
- Step 2: 94°C, 30 seconds
- Step 3: 55°C, 30 seconds
- Step 4: 72°C, 30 seconds, repeat steps 2-4 30 times
- Step 5: 72°C, 10 minutes

The PCR reactions were performed with the purpose of changing a 5'end NcoI-restriction site to an NdeI-restriction site by using three different 5'end primers (the 3'end primer was the same in all reactions). In total three nucleotides were changed, one in each reaction.

PCR screening

Four different PCR's were performed on genomic DNA to verify transformants at the DNA level. The settings were similar to the PCRs performed in the subcloning, except for the annealing temperature (step 3), and for some of the primersets the elongation time (step 4). The primer sequences can be found in Appendix III, together with the annealing temperature for each reaction. Elongation time is written if the time length differs from 30 seconds.

Primerset	Primer 1	Primer 2
1	5'NUD3'-5comp	3'psaDterm
2	5'NUD3'-5comp	NUD3'
3	5'NUD GFP	3'psaD495
4	5'paro4331	3'paro4743

Table 3.1 Overview of the different primers used for screening. The table shows the different primersets used to verify the positive transformants at the DNA level.

3.1.5 Restriction digestion of DNA

Restriction enzymes were used according to the manual achieved from the manufacturers, Promega and New England Biolabs, to cut DNA. The conditions (restriction buffer and requirement of BSA) differed according to the enzymes used in the different reactions.

Vector DNA

The DNA was cut in a 50µL solution with 5µL restriction buffer [10X] and if required 5µL BSA [1mg/mL]. 10µg DNA was cut with 20U of each enzyme for at least 1 hour.

Genomic DNA (Southern blot)

The DNA was cut in a 50µL solution with 5µL restriction buffer[10X] and if required 5µL BSA[1mg/mL]. 3.4µg DNA was cut with 20U of each enzyme for at least 4 hours.

3.1.6 Ligation

The ligation was performed in a 10 µL reaction.

Procedure:

- Mix insert-DNA and vector-DNA and add dH₂O up to 6.5µL. Incubate 5 minutes at 45°C.
- Cool to room temperature and add 1µL T4 DNA ligase buffer [10X].
- Add 2µL polyethylene glycol 8000 [30%, w/v].
- Add 0.5µL T4 DNA ligase enzyme (3 u/µl, Promega).
- Incubate for 3 hours at 19°C.

After incubation, the ligation-mixture was kept at room temperature for 30 minutes before the mixture immediately was used in transformation. An insert: vector ratio of 1.3: 1 was used in all ligations (inserts of 0.5-3 kb).

3.2 Subcloning

3.2.1 Plasmids

pEcoli_nudix

The subcloning started with PCR from a vector named *pEcoli_nudix*. This is a *pEcoli-Cterm6xHN* vector (Clontech) where the *Nudix* gene has been cloned into the *XbaI* and *NcoI* restriction sites of the plasmid (performed by Uwe Klein), adding a histidine-tag at the C-terminal end of *rppH*.

Sk+_mod

The *rppH* gene was further cloned into an intermediate vector called *Sk+_mod*. This is a Bluescript II *Sk+* vector (Stratagene) which was modified prior to the subcloning by insertion of the *XhoI-XbaI* sequence from the *pChlamiRNA3int* vector (Chlamydomonas resource center, Minnesota) by *XhoI/XbaI* restriction cutting and ligation. The gene was inserted between the *NdeI* and *XbaI* restriction sites.

-pChlamiRNA3int

The *pChlamiRNA3int* vector (Chlamydomonas resource center, Minnesota) is described by Molnar et al. (2009).

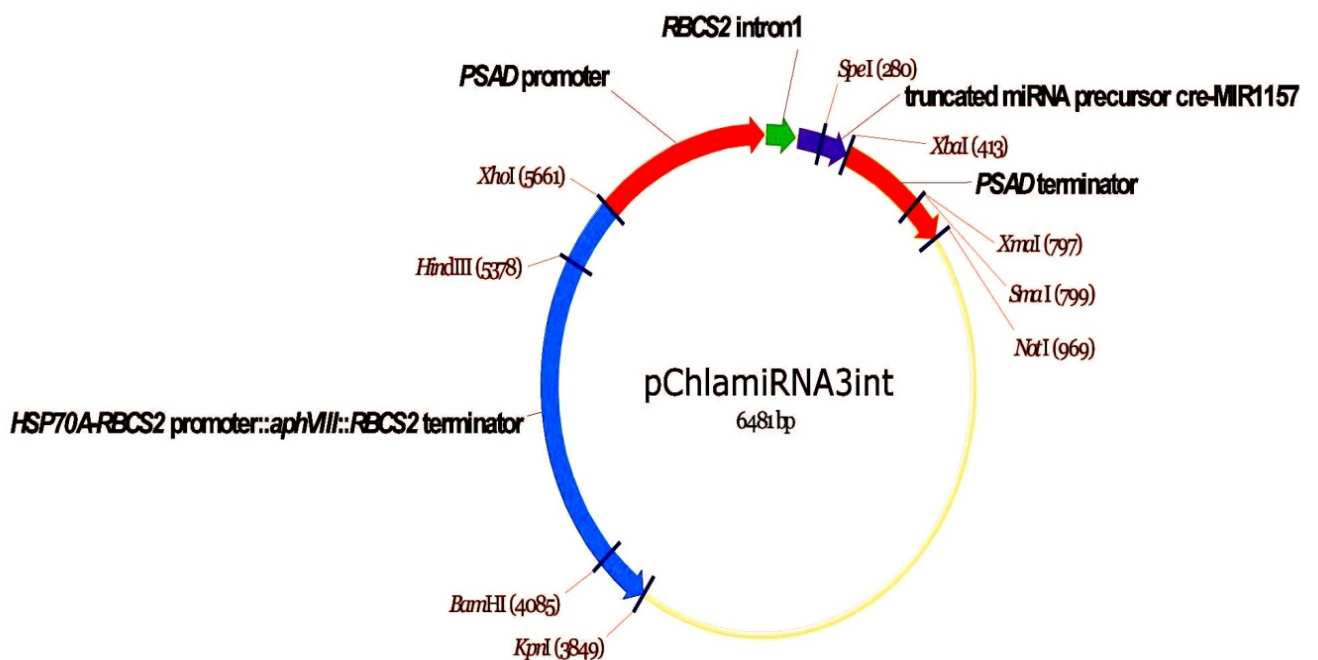


Figure 3.1 The *pChlamiRNA3int* vector. Figure taken from <http://chlamycollection.org/plasmid/pchlamirna3int/>.

3.3 Work with *Escherichia coli* (*E. coli*)

3.3.1 *E. coli* strain

Competent *E. coli* TB1 cells (NEB) were used in the cloning. For expressing proteins *E. coli* BL21 cells were used.

3.3.2 Growth of *E. coli*

E. coli was grown in liquid lysogeny broth (LB) media, in liquid lysogeny broth (LB) media with ampicillin or on lysogeny broth plates (LB) with ampicillin at 37°C.

Liquid (LB) media

Mix 10 g tryptophan, 5 g yeast extract, 10 g NaCl, 200µL 5M NaOH in 1 liter H₂O. Sterilize by autoclavation.

Liquid (LB) media with ampicillin

For selection after transformation, the *E. coli* cells were grown in 100mL LB medium with ampicillin [60µg/mL]. Ampicillin was added after autoclavation.

Solid (LBA) media

LB media with 1.5% agar and 60µg/mL ampicillin. Ampicillin was added after autoclavation.

Ampicillin 60mg/mL

Mix 600mg ampicillin in 10mL dH₂O. Sterilize the solution with filtering through 0.22µm filtes.

3.3.3 Preparation of competent *E. coli* cells

TB1 *E. coli* cells were made competent by CaCl₂ treatment.

Procedure:

- Grow TB1 *E. coli* cells in LB medium (3mL) overnight on rotating weel at 37°C.
- Inoculate 100mL LB medium with 2mL of the culture. Grow at 37°C on shaker.
- After approximately 2.5 hours, when O.D₆₀₀ is around 0.6, the cells should be harvested in two 50mL capped plastic tubes (5000rpm for 10 min at 4°C).
- Resuspend the pellet in each tube in 20mL ice-cold sterile 0.1M CaCl₂. Leave on ice for 10 min.
- Spin tube as previously and resuspend the pellet in ice-cold sterile 2mL 0.1M CaCl₂ with 15% glycerol.
- Store the cells in 1.5mL microfuge tubes in 50µL aliquots. The cells should be frozen immediately, either in liquid nitrogen or in a -80°C freezer.

3.3.4 Transformation of competent *E. coli* cells

- Melt an aliquot of frozen competent cells (50µL) on ice and add 3µL of pure DNA (1ng/µL) or 3µL of ligation reaction mixture.
- Keep the mixture on ice for 30 minutes.
- Heat shock the cells for 90 seconds at 42°C. Cool down immediately on ice. Transfer the cells into a 15mL loose cap tube.
- Add 0.8mL LB medium and incubate at 37°C for 45-60 minutes on a rotating wheel.
- Plate out 75µL cells on a LB plate with ampicillin [60µg/mL].
- Incubate at 37°C for 16-24 hours. The colonies should be picked immediately or stored at 4°C.

3.3.5 Culturing of *E. coli* on agar plate colonies

A single colony is picked with a sterile pipet tip from the plate and put into a 15mL loose cap tube containing 3mL LBA medium (LB medium with ampicillin [60µg/mL]). The cells are grown overnight at 37°C with rotation until stationary phase. Next day the tube can be used in plasmid miniprep isolation.

3.3.6 Storage of *E. coli* at -80°C

0.7mL of *E. coli* culture is mixed with 0.3mL of 50% glycerol. The cells are then stored in the freezer at -80°C.

3.3.7 Plasmid isolation from *E. coli*

Miniprep plasmid isolation

For small-scale plasmid isolation mini-preps were performed according to the following protocol.

Procedure:

- Take 1.5mL of *E. coli* culture (3.3.5) and harvest the bacteria by centrifugation in a 1.5mL microfuge tube (13000g for 30sec at 4°C).
- Resuspend the bacteria in 100µL ice-cold TEG buffer and leave the mixture in room temperature for 5 minutes.
- Add 200µL of NaOH/SDS [0.2N, 1% (w/w)] solution prepared fresh. Mix by inversion and incubate on ice for 5 minutes.
- Add 150µL ice-cold potassium acetate [5M potassium, 3M acetate], mix by inversion and incubate on ice for 5 minutes.
- Centrifuge at 13000g for 5 minutes at 4°C, and transfer the supernatant to a new 1.5mL tube.
- Add 410µL of phenol/chloroform/isoamyl alcohol [25:24:1], and mix by vortexing.
- Centrifuge at 13000g for 2 minutes, and transfer the upper phase to a new 1.5mL tube.
- Add 410µL chloroform/isoamyl alcohol [24:1], mix by vortexing and centrifuge at 13000g for 2 minutes.

- Transfer 310µL of upper phase to a new 1.5mL tube, add 750µL of ice-cold ethanol [96%], mix by vortexing and incubate on ice for 10 minutes.
- Centrifuge at 13000g for 10 minutes at 4°C, discard supernatant and wash the pellet with 1mL of [70%] ethanol.
- Centrifuge at 13000g for 5 minutes at 4°C, discard supernatant and dry the pellet in a vacuum centrifuge or by leaving it at room temperature.
- Resuspend the pellet in 15µL of dH₂O.

Expected yield is 1 to 3µg DNA when isolating plasmid from 1.5mL cell culture.

Maxi-prep

Maxi-prep was performed for large scale plasmid isolation, and a CsCl density gradient centrifugation method was used according to the following protocol.

Procedure:

- Inoculate 100mL LBA liquid media with 5µL of an *E. coli* culture or one *E. coli* colony from a plate, and grow culture overnight at 37°C with shaking until stationary phase.
- Harvest the cells by centrifugation (6000g at 4°C for 5 minutes) and resuspend the bacteria in 3.6mL ice-cold TEG buffer.
- Add 0.4mL of lysozyme [10mg/mL] in TEG prepared fresh, and leave in room temperature for 5 minutes, before moving the tube to an ice bath for 5 additional minutes.
- Add 8mL NaOH/SDS [0.2N, 1% (w/w)] solution prepared fresh, mix by inversion and leave on ice for 5 minutes.
- Add 6mL ice-cold potassium acetate [5M potassium, 3M acetate], mix by inversion and incubate on ice for 5 minutes.
- Centrifuge at 6000g for 10 minutes at 4°C.
- Transfer supernatant to a new tube (by using cheesecloth) and add 12.5mL isopropanol. Mix by vortexing and leave for incubate at room temperature for 15 minutes.
- Centrifuge at 6000g for 10 minutes (room temperature).
- Discard supernatant and let the pellet dry in room temperature.
- Resuspend the pellet in 3mL TE [50mM Tris (pH 8), 1mM EDTA] buffer. Add TE [50mM Tris (pH 8), 1mM EDTA] buffer until the solution has a weight of 4.2g.
- Add 4.5g CsCl to the solution, mix and let the solution warm up to room temperature.
- Add 0.5mL ethidium bromide [10mg/mL], mix and centrifuge at 6000g for 5 minutes (room temperature).
- Transfer supernatant to a Beckman OptiSeal tube. Centrifuge at 50000g, 15°C for 15 hours or more using a VTi 65.2 rotor. The rotor should be decelerated without brake.
- Illuminate tube in 350nm UV light and extract the middle band containing the plasmid DNA using a 2mL syringe.
- Extract the solution with 0.75mL isopropanol/water [7:1] up to five times to remove the ethidium bromide.
- Dialyze the solution against 200mL TE [50mM Tris (pH 8), 1mM EDTA] buffer at 4°C to remove CsCl. The buffer should be replaced every hour, twice or three times.
- The DNA concentration can be determined by measuring O.D_{260nm} on a 1:100 diluted sample (chapter 3.1.3).

The DNA can be stored in a -20°C freezer. From 100mL cell culture a yield of about 150 to 400µg of DNA is expected.

3.3.8 Protein expression

Protein expression was done in *E. coli* BL21 cells. Before expression the cells were transformed as described in section 3.3.4.

Procedure:

- Grow one colony of transformed BL21 *E. coli* cells in LBA medium (3mL) overnight on rotating wheel at 37°C.
- Inoculate 100mL LBA medium with 2mL of the culture. Grow at 37°C on shaker.
- After approximately 2.5 hours, when O.D₆₀₀ is around 0.6, add 112µL IPTG (200ng/µL).
- After approximately 6 hours the cells can be harvested by centrifugation (5 minutes, 5000rpm).
- Wash the pellet by resuspending the cells in water and harvest them by centrifugation (5 minutes, 5000rpm).
- Resuspend the pellet in buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole). Use immediately or store the cells in a -80°C freezer.

3.3.9 Preparation of Cobalt sepharose column

The sepharose used in the experiment was TALON®Metal Affinity resin (Clontech).

Procedure:

- Shake the bottle containing the sepharose until the solution is homogenous.
- Add 0.5mL sepharose to a 2mL microfuge tube.
- Centrifuge at 500g for 5 minutes.
- Discard the supernatant, add 1.5mL dH₂O, and mix for 3 minutes.
- Centrifuge at 500g for 5 minutes.
- Discard the supernatant, add 1.5mL buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole), and mix for 3 minutes.
- Centrifuge at 500g for 5 minutes.
- Discard the supernatant and add buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole) until the final volume is around 0.5mL.
- Carefully place the sepharose on the column and use immediately.

3.3.10 Isolation of proteins from *E. coli* (affinity chromatography)

Procedure:

- Thaw transformed protein expression cells (stored in a -80°C freezer) on ice and sonicate the cells in a Vibra M Cell™ (Sonics) with 4 x 5 seconds pulses in a cold rack.

- Centrifuge the samples at 4°C at maximum speed for 10 minutes, and filtrate the supernatant through a Sterile Acrodisc 0.2µm (Gelman Sciences) to avoid bacteria debris. Collect a small sample from the supernatant (crude extract).
- Load the rest of the supernatant on a Cobalt sepharose column (TALON Metal Affinity Resin from Clontech).
- Collect a sample of the flow through (flow through).
- Wash the column twice with 3mL buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole). At the end of the last wash, collect a sample (wash).
- Elute the protein by adding 0.5mL of buffer B ([20mM] Na-phosphate, [500mM] NaCl, [500mM] imidazole pH 7.4) to the column. Collect everything after the first two drops (elute). The samples can be analyzed with SDS-PAGE.

3.4 Work with *C. reinhardtii*: Algal strain, media and methods

3.4.1 *C. reinhardtii* strain

The mutant strain cw15 of *C. reinhardtii* was obtained from the *Chlamydomonas* genetic center at the University of Duke NC, USA.

3.4.2 Media for growing *C. reinhardtii*

Liquid high salt (HS) media (1 liter)

- Mix 20mL of phosphate solution [50X], 20mL of high salt solution [50X] and 1mL of Trace elements.
- Fill up with water to 1L and sterilize by autoclavation.

Solid media

- Prepare 1L of liquid HS media with 1.5% agar and sterilize by autoclavation.
- Cool solution to 50°C and add paromomycin to a final concentration of 60µg/mL.
- Pour the solution into plastic plates and let solidify.

3.4.3 Nuclear transformation of *C. reinhardtii*

Nuclear transformation is performed with glass bead agitation on cell wall-less cells, a method developed by K.Kindle (Kindle 1990).

Procedure

- Make a 30% PEG 8000 (Sigma) stock solution. Sterilize the solution together with glass beads (0.45-0.52mm in diameter (Sigma) by autoclavation.
- Prepare the final vector in dH₂O to a final concentration of 1µg/µL.
- Grow cw15 cells until a concentration of 1-2 x 10⁶ cells/mL.
- Harvest the cells by centrifugation at 5000g. Resuspend the pellet in HS medium until a concentration of 300x10⁶ cells/mL.

- Add 300mg sterilized glass beads (Sigma) and 5µg DNA to 0.334mL cells [300×10^6 cells/mL] and 0.066mL PEG [30%] in a 15mL canonical disposable polypropylene centrifuge tube.
- Agitate cells for 10 seconds at top speed on a Fisher Vortex Genie II mixer.
- Allow the beads to settle and spread the cells on a HS plate with paromomycin.
- Let the plates dry for 24 hours in room temperature with constant light.
- Turn the plates around and seal with parafilm.

3.4.4 Growth conditions for transformed *C. reinhardtii*

After transformation, *C. reinhardtii* was grown under continuous light on HS agar plates containing paromomycin. It took approximately three weeks until colonies were visible on the plates. 50 colonies were then transferred to a new HS agar plate with paromomycin containing a grid. In fear of too low paromomycin concentration on the plate, this step was repeated. After two weeks on the plate, the growing colonies were large enough to be picked and inoculated into 200 mL liquid HS medium. The liquid cultures were grown under continuous light. To improve growth, the cultures were after a week transferred to a 100mL tube, and grown with continuous light and bubbling with 2% CO₂-enriched air in a 32°C water bath.

3.4.5 Total DNA isolation from *C. reinhardtii*

The cells were first grown to an approximate concentration of 2×10^6 cells/ml.

Procedure:

- Harvest 40-50mL of cell culture by centrifugation at 5000g at 4°C for 5 minutes.
- Resuspend the pellet in 0.75mL DNA extraction buffer [100mM Tris pH 8.0, 50mM Na₂-EDTA, 0.5M NaCl, 10mM β-mercaptoethanol], and transfer to a 2mL microfuge tube.
- Add 60µL SDS [21% w/v] and incubate for 15 minutes at 65°C.
- Cool down to room temperature and add 0.9mL phenol (equilibrated with 0.1M Tris pH 8.0), mix by inversion.
- Centrifuge at 13000g for 5 minutes and transfer 750 µL of upper phase to new 2mL microfuge tube.
- Add 750µL phenol/chloroform/isoamylalcohol [25:24:1] and mix by inversion.
- Centrifuge at 13000g for 5 minutes and transfer 650µL of upper phase to new 1.5mL microfuge tube.
- Add 650µL isopropanol, mix by inversion, and incubate at room temperature for 5 minutes (or until precipitate occurs).
- Collect the nuclear acids by centrifugation at 2000g for 1 minute. Add 1mL of ethanol [70%].
- Centrifuge at 13000g for 2 minutes, discard the supernatant and dry the pellet in room temperature or in a vacuum centrifuge.
- Resuspend in 90µL TE buffer [10mM Tris pH 8.0, 1mM Na₂EDTA], add 10µL RNase A [1mg/mL], and incubate at 37°C for 1 hour.
- Extract the mixture, once with 100µL phenol/chloroform/isoamylalcohol [25:24:1] and once with 100µL chloroform/isoamylalcohol [24:1].
- Precipitate the DNA on ice for 1 hour with ethanol [96%] and Sodium acetate [pH5.2, 3M] (1/10th volume of sodium acetate and two volumes of ethanol).

- Centrifuge at 13000g for 10 minutes at 4°C to collect the precipitate, and wash the pellet with 1mL ethanol [70%].
 - Centrifuge at 13000g for 5 minutes at 4°C, discard the supernatant and dry the pellet in a vacuum centrifuge or at room temperature.
 - Resuspend the DNA in 20µL dH₂O, and measure concentration by dot spot.
- A concentration of 200ng/µL can be expected. The DNA can be stored in a -20°C freezer.

3.4.6 RNA isolation from *C. reinhardtii*

All work was done with RNase-free consumables and solutions. The cells were grown with 12-hour light/12-hour dark cycle to an approximate concentration of 2x10⁶ cells/ml prehand.

Procedure:

- Harvest 40-50mL cell culture by centrifugation at 5000g for 5 minutes at 4°C.
- Resuspend the pellet in 1.5mL ice-cold lysis buffer [0.6M NaCl, 200mM Tris pH 8.0, 10mM Na₂EDTA] and add 150µL RNase inhibitor [200mM vanadyl ribonucleoside (NEB)].
- Transfer the solution to a 15mL tube preheated to 65°C containing 2mL phenol (equilibrated with 0.1M Tris pH 8.0) and 1.5mL SDS [4%].
- Incubate for 15 minutes at 65°C, mix occasionally by shaking.
- Cool down on ice and add 1mL ice-cold chloroform/isoamylalcohol [24:1].
- Centrifuge at 8000g for 5 minutes at 4°C.
- Transfer 3mL of the upper phase to a new 15mL tube and add 3mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1].
- Centrifuge at 8000g for 5 minutes at 4°C.
- Transfer 2.5mL of the upper phase to a new 15mL tube and add 2.5mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1].
- Centrifuge at 8000g for 5 minutes at 4°C.
- Transfer 2.0mL of the upper phase to a new 15mL tube and add 2.0mL ice-cold isopropanol and 250µL Sodium acetate [3M, pH 5.2]. Incubate at -20°C for at least 1 hour.
- Centrifuge at 12000g for 15 minutes at 4°C, discard the supernatant and invert the tube to completely dry the pellet.
- Resuspend the pellet in 300µL DEPC-treated water, transfer to a 1.5mL microfuge tube and add 100µL ice-cold LiCl [3M]. Incubate on ice for 2 hours.
- Centrifuge at 13000g for 30 minutes at 4°C.
- Discard the supernatant and resuspend the pellet in 100µL DEPC-treated water.
- Determine the concentration by diluting 10µL of the solution in 1mL DEPC-treated water and measure O.D_{260nm}.
- Precipitate the RNA in the rest of the solution with 10µL Sodium acetate [3M, pH 5.2] and 200µL ethanol [96%]. Incubate at -20°C for 1 hour.
- Centrifuge at 13000g for 10 minutes at 4°C to collect the precipitate, and wash the pellet with 1mL ethanol [70%].
- Centrifuge at 13000g for 10 minutes at 4°C, discard the supernatant and dry the pellet in a vacuum centrifuge or at room temperature.
- Resuspend the RNA to a concentration of 2µg/µL in DEPC-treated water.

3.4.7 Chloroplast isolation from *C. reinhardtii*

Chloroplasts were isolated from *C. reinhardtii* with a Percoll gradient. The method was developed by Mason and coworkers (Mason et al. 2006).

Procedure:

- Inoculate cells at a density of 4×10^4 cells/mL in 1 liter of HS media.
- Grow cells in continuous light. After two days switch to a 12-hour light/12-hour dark cycle until the cells have an approximate concentration of $0.6-1.0 \times 10^7$ cells/ml.
- Harvest the cells at the fourth hour into the light cycle by centrifugation (3000g, 10 minutes, 4°C). Wash the cells with 100mL of 50mM HEPES-KOH pH 7.5, centrifuge at 3000g for 5 minutes at 4°C, and resuspend the pellet in 2mL 50mM HEPES-KOH pH 7.5. Hold the cells at 4°C.
- Determine the chlorophyll concentration of the cells spectrometrically (should be between 1.5-2.0mg chlorophyll/ml. Perform all remaining steps at 4°C.
- Add 8-10mL isolation buffer with 1%BSA (w/v), and immediately draw the dilution into a syringe. Attach a 27-gauge needle and break the cells by passing them through the needle at a flow rate of 0.1mL/s. Do not keep the cells in isolation buffer for more than a few minutes during the cell breakage, since this would decrease the yield of intact chloroplasts significantly.
- Centrifuge for 2 minutes at 750g at 4°C to collect intact cells and chloroplasts.
- Gently resuspend the cells in 2mL isolation buffer with a cut 1mL pipet tip (if the diameter is too small the chloroplasts will break). Clumps will destroy the gradient and should be avoided.
- Overlay the solution on top of the gradients and centrifuge (4200g for 15 minutes). The thylakoids should predominantly be in a band at the 20-45% interface, intact chloroplasts should be in a band at the 45-65% interface, while unbroken cells should form a pellet at the bottom of the tube.
- Collect the band containing the intact chloroplasts by using a disposable glass pipet. Verify the isolated chloroplasts by checking in a microscope.

For solutions and Percoll gradient recipe, see Appendix IV. Affinity chromatography, SDS-PAGE, and mass spectrometry can further be performed to analyze the amount of the RppH in chloroplasts.

3.4.8 Quantification of RNA and chlorophyll

Spectrometry measurements were used to estimate RNA and chlorophyll concentration.

RNA concentration

Absorption was measured at $O.D_{260nm}$ after isolation of total RNA.

$$O.D. 1.0 = 40\mu\text{g RNA/mL}$$

Chlorophyll concentration

Absorption was measured at $O.D_{663nm}$ and $O.D_{645nm}$ in 80% acetone. The concentration was calculated as follows:

$$(A_{663nm} * 8.02) + (A_{645nm} * 20.2) = \mu\text{gChl/mL in cuvette.}$$

3.4.9 Protein isolation from *C. reinhardtii*

C. reinhardtii was grown to an approximate concentration of 2×10^6 cells/mL with 12-hour light/12-hour dark cycle. The proteins were isolated from *C. reinhardtii* four hours into the light according to the following procedure:

- Spin down approximately 150mL cell culture (4000g for 5 minutes), wash the cells in buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole) and spin as previous.
- Resuspend the cells in 1mL buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole) containing protease inhibitor (1 tablet Protease Inhibitor Cocktail Tablet (Roche) in 10mL buffer).
- Sonicate the cells until they are broken (check regularly in a microscope) in a Vibra M Cell™ (Sonics) with 4 x 5 seconds pulses in a cold rack.
- Centrifuge the samples at 4°C at maximum speed for 10 minutes, and filtrate the supernatant through a Sterile Acrodisc 0.2µm (Gelman Sciences) to avoid cell debris. Collect a small sample from the supernatant (crude extract).
- Load the rest of the supernatant on a Cobalt sepharose column (TALON Metal Affinity Resin from Clontech).
- Collect a sample of the flow through (flow through).
- Wash the column twice with 3mL buffer A ([20mM] Na-phosphate, [500mM] NaCl, [20mM] imidazole). At the end of the last wash, collect a sample (wash).
- Elute the protein by adding 0.5mL of buffer B ([20mM] Na-phosphate, [500mM] NaCl, [500mM] imidazole) to the column. Collect everything after the first two drops (elute). The samples can be separated with SDS-PAGE.

3.5 Analytical methods

3.5.1 Sequencing

The final transformation vector was sequenced by Eurofins MWG/Operon (Germany) to verify the construct. The primers used were designed to sequence the *rppH* sequence and some of the 5' and 3' flanking regions.

3.5.2 Reverse transcription

A RevertAid™ H Minus First cDNA Synthesis Kit (Fermantas) was used.

Procedure:

- Mix 10ng-5µg of total RNA and 15-20pmol of a sequence specific primer on ice. Add DEPC water to 12µL. Mix gently and spin down.
- Incubate at 70°C for 5 minutes, cool down on ice and centrifuge briefly.
- Add 4µL reaction buffer [5X], 1µL RiboLock™ Ribonuclease Inhibitor [20u/µL] and 2µL dNTP mix [10mM] on ice. Mix gently and centrifuge to collect drops.
- Add 1µL RevertAid™ H Minus M-MuLV Reverse transcriptase [200u/µL].
- Incubate mixture at 42°C for 1 hour.

-Stop the reaction by heating the mixture to 70°C for 10 minutes. The first strand cDNA can be amplified directly by PCR.

3.5.3 DNA and RNA blots

3.5.3.1 Preparation of radioactive probes

A radioactive probe was made by mixing DNA template, DNA polymerase, random hexanucleotide primers, and dNTPs. The deoxycytidine triphosphate in the dNTP mix is marked with a radioactive ³²P-phosphorus isotope. The isotope is incorporated at the α-phosphate ([α-³²P]-dCTP), resulting in a high energy β-particle emission with a half-life of 14.3 days.

Procedure:

-Perform a PCR, amplifying a sequence of interest. The amplification product will be used as DNA template for the radioactive probe.

-Mix 10.5μL dH₂O and 2μL template DNA [100-200ng/μL].

-Keep the tube on boiling water for at least 5 minutes for denaturation.

-Add 5μL cold labeling buffer, 2.5μL BSA [4mg/mL], 1.5μL dNTP mixture (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate [1mM of each mixed 1:1:1]), and 2.5 μL (25 microcurie (μCi)) [α-³²P]-dCTP. Mix.

-Add 1 μL DNA polymerase I, (Klenow fragment) [2 units/mL] (NEB).

-Incubate at room temperature for 3 hours.

The solution can be stored at -20°C.

3.5.3.2 Southern analysis

Procedure:

-Isolate genomic DNA as described (3.4.5)

-Digest 3.4μg of genomic DNA (see section 3.1.5).

-Precipitate the DNA on ice for 1 hour with ethanol [96%] and Sodium acetate [3M, pH 5.2]. (1/10th volume of sodium acetate and two volumes of ethanol.)

-Centrifuge at 13000g for 10 minutes at 4°C to collect the precipitate, and wash the pellet with 1mL ethanol [70%].

-Centrifuge at 13000g for 5 minutes at 4°C. Remove the supernatant.

-Resuspend the DNA in 10μL of TAE-buffer [1X] and add 2μL gel loading buffer.

-Separate the DNA with agarose gel electrophoresis on a 1% agarose gel (described in chapter 3.1.1).

-Take a picture of the gel under UV-light.

-Denature the DNA by incubating the gel for 30 minutes in denaturing solution.

-Neutralize the gel by incubating it for 30 minutes in neutralizing solution.

-Fill a capillary transfer apparatus with SSC [10X] solution. Prewet three 3mm thick Whatman papers and place them in the transfer apparatus.

-Place the gel in inverted orientation on top of the Whatman papers. Place a prewetted nylon membrane on top of the gel, be careful to remove bubbles.

- Place two prewetted 3mm Whatman papers, a stack of thin papers, and a stack of paper towels on top of the membrane in the written order.
- Transfer the DNA overnight.
- Rinse the membrane in SSC [2X], and crosslink the DNA to the membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
- Hybridize the membrane with a probe and visualize the result by autoradiography.

3.5.3.3 Northern analysis

It is important that all the equipment used is RNase-free.

Procedure:

- Sterilize a gel tray, a comb and an electrophoresis chamber with H₂O₂ [3%] overnight.
- Prepare a 1.3% agarose gel by dissolving 0.78g agarose in 37mL DEPC-treated water. Add 12mL MOPS [5X] and 11mL formaldehyde [37%] when the solution has cooled down to 65°C.
- Prepare samples by mixing following solutions;
 - 2.0μL MOPS buffer [5X]
 - 3.5μL formaldehyde
 - 3.5μL ethidium bromide [100μg/mL]
 - 10.0μL formamide
 - 4.5μL RNA [2μg/μL]
- Incubate the samples for 15 minutes at 65°C.
- Put the gel into the electrophoresis chamber with running buffer (90mL MOPS [5X], 315mL DEPC-treated water, 45mL formaldehyde) at the end of the incubation period. Apply power (60mA) to the gel for 5 minutes.
- Cool down samples, spin shortly and add 2.0μL RNA-gel loading buffer.
- Load 20μL sample in each well and fractionate the samples, first in reverse direction for 10 minutes (60mA), then in normal direction until the bromphenol blue band is at the bottom of the gel (60mA).
- Wash the gel briefly in DEPC-treated water and take a picture under UV-light.
- Fill a capillary transfer apparatus with RNA transfer solution. Prewet three 3mm thick Whatman papers and place them in the transfer apparatus.
- Place the gel in inverted orientation on top of the Whatman papers. Place a prewetted nylon membrane on top of the gel, be careful to remove bubbles.
- Place two prewetted 3mm Whatman papers, a stack of thin papers, and a stack of paper towels on top of the membrane in the written order.
- Transfer the RNA for 6 hours.
- Take a photo of the membrane. Check gel for complete transfer.
- Rinse the membrane in SSC [2X], and crosslink the DNA to the membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
- Hybridize the membrane with a probe and visualize the result by autoradiography.

3.5.3.4 Hybridization

The protocol is used for hybridization using a random primer labeled DNA probe.

Procedure:

- Preheat the hybridization buffer to 65°C in a water bath.
- Wash the blotting membrane in dH₂O.
- Prehybridize the blotting membrane for at least 15 minutes in 1mL of hybridizing buffer at 65°C with rotation.
- Discard hybridization buffer and add 0.1mL hybridization buffer per square cm of the blotting membrane.
- Add random primer labeled probe until the volume corresponds to 1:2000 of the hybridization buffer.
- Incubate at 65°C for about 24 hours with rotation.
- Discard the radioactive hybridization buffer.
- Wash the membrane twice with preheated (to 65°C) wash buffer #1 for 5 minutes at 65°C with rotation.
- Wash the membrane six times with preheated (to 65°C) wash buffer #2 for 5 minutes at 65°C with rotation. An additional wash with wash buffer #2 for 20 minutes completes the washing.
- Wrap the membrane in plastic, investigate the radioactivity with a Geiger counter, and visualize the result by autoradiography.

3.5.3.5 Autoradiography

Kodak®BioMax®MS equipment and solutions were used.

Procedure:

- Place the plastic wrapped membrane in an autoradiography cassette with an intensifying screen.
- Expose to an x-ray film at -80 °C for a few hours, overnight or for several days, depending on the Geiger counter investigations (3.5.3.4.).
- Develop and fix the film in a dark room.

3.5.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Preparing polyacrylamide gel:

- A 12% polyacrylamide solution was prepared by adding 1.70mL dH₂O, 1.37mL solution A, 1.03mL solution B, and 16.25µL ammonium persulfate [10%] in a 15mL tube.
- Mix gently and immediately pour the solution into assembled glass plates on the casting frame until 2cm below the rim of the small plate.
- Gently overlay the solution with dH₂O and let solidify for 30 minutes.
- Remove the water and prepare a 4% stacking gel by mixing 1.80mL dH₂O, 0.40mL solution A, 0.75mL solution B', and 30µL of ammonium persulfate [10%].

-Mix gently and immediately pour the solution on top of the 12% gel in the assembled glass plates. Fill the space between the glass plates completely with the solution and insert a comb. Let solidify for 30 minutes.

Electrophoresis:

-Place the gel in an electrophoresis chamber and fill the chamber with electrophoresis running buffer [1X].

-Mix 20 μ L of each of your samples with 10 μ L SDS loading buffer [3X]. Denature the samples at 95°C for 5 minutes and cool down on ice.

-Load 10 μ L sample per well. For estimating the size of the protein-samples, a SDS-PAGE molecular weight standard solution (Bio-Rad) was used.

-Separate the proteins for 15 minutes at 80V followed by 1 hour at 120V.

-Continue with Western blot or stain the gel with staining solution for 15 minutes on a shaker. Destain the gel with destaining solution overnight.

3.5.5 Western blot and antibodies

3.5.5.1 Western blot

SDS-PAGE is performed in advance. The procedure can be divided into three steps; 1) transferring, 2) blocking and incubation, and 3) detection.

Transferring

Procedure:

-Soak the SDS gel containing your samples, a nitrocellulose membrane (Bio-Rad), two fiber pads and two filter papers (Bio-Rad) in transfer buffer for a few minutes.

-Open a transfer apparatus gel cassette and place it in a tray filled with transfer buffer. The black panel of the cassette should be placed towards the bottom of the tray.

-Prepare the transfer sandwich in the following order on the black panel of the cassette:

1) Fiber pad, 2) filter paper, 3) SDS gel, 4) nitrocellulose membrane, 5) filter paper, 6) fiber pad.

-Be careful to assemble the cassette when it is completely soaked in the buffer to avoid bubbles.

-Insert the gel cassette into an electrode module. Let the black panel face the black cathode electrode panel.

-Place a cooling unit in the transfer and fill up with transfer buffer.

-Transfer for 10 minutes at 100V, then 20 minutes at 60V, all the time with constant stirring.

-Stain the membrane with Ponceau S ([1%] in [1%] acetic acid) and destain it with acetic acid [1%] to check for complete transfer. Additionally the SDS-gel can be stained with Croomassive blue.

Blocking and incubation:

Procedure:

-Incubate the nitrocellulose membrane containing your protein samples with blocking buffer on a shaker for 1-2 hours at 37°C or overnight at 4°C.

-Dilute the primary antibody in primary antibody dilution buffer. For dilutions used for the different antibodies see section 3.5.5.2. Incubate the membrane with the diluted primary antibody on a shaker for 1 hour at 37°C or overnight at 4°C.

-Wash the membrane four times with washing buffer on a shaker 5-10 minutes each time. (Note: For HRP conjugated primary antibody, go directly to detection and drop the two following steps.)

-Dilute the secondary antibody in blocking buffer. For dilutions used for the different antibodies see section 3.5.5.2. Incubate the membrane with the diluted secondary antibody on a shaker for 1 hour at 37°C or overnight at 4°C.

-Wash the membrane four times with washing buffer on a shaker 5-10 minutes each time.

Detection:

Luminata™ Forte Western HRP substrate (Millipore) was used for detection.

Procedure:

-Place your membrane with the protein side up and add the Luminata solution, 0.1mL solution per cm² membrane. Incubate for 2-5 minutes.

-Drain off excess liquid, wrap in plastic and expose to film within a cassette.

-Expose the film between 1-5 minutes.

-Develop and fix the film by using Kodak®BioMax®MS equipment and solutions in a dark room.

3.5.5.2 Antibodies

In total three different sets of antibodies were used for detection of GFP and his-tagged proteins.

Detection of histidine-tagged proteins:

Antibodies

	Primary antibody		Secondary antibody	
	Name	Product/cat. Number	Name	Product/cat. number
Set 1	His Tag Antibody (Anti-His/clone HIS.H8/EH158) (BioSite)	OAEA00010	Anti-MOUSE IgG2b (Gamma 2b chain) Antibody (RABBIT) Peroxidase Conjugated (BioSite)	OARA05036
Set 2	THE™ His Tag Antibody [HRP], mAb, Mouse (GenScript)	A00612	NONE	

Table 3.2 Antibodies in set 1 and 2. Table showing the different antibodies used for detection of the histidine-tag in RppH-6xHN.

Dilutions

	Primary antibody	Secondary antibody
Set 1	3,513889	0,388889
Set 2	0,736111	

Table 3.3 Antibody dilutions. Table showing the dilutions used for the different antibodies in set 1 and 2.

Detection of GFP:

Antibodies

	Primary antibody		Secondary antibody	
	Name	Product/cat. number	Name	Product/cat. number
Set 3	Living Colours® Full-Length ZsGreen Poluclonal antibody (Clontech)	632474	Anti-Rabbit IgG (whole molecule)- Peroxidase (Sigma-aldrich)	A0545

Table 3.4 Antibodies in set 3. Table showing the different antibodies used for detection of GFP in 5'RppH-GFP-6xHN.

Dilutions

	Primary antibody	Secondary antibody
Set 3	0,736111	1:10 000

Table 3.5 Antibody dilutions. Table showing the dilutions used for the different antibodies in set 3.

3.5.6 Mass spectrometry

The procedure can be divided in three steps 1) In-gel digest procedure, 2) MS Analysis, and 3) Data analysis.

In gel digest procedure, received from Anders Moen, UiO.

SDS-PAGE with the specific samples is performed prehand. The band is further cut out of the gel and sliced into smaller pieces which are transferred to a 1.5mL microfuge tube and digested with trypsin according to the following protocol.

Procedure:

- Add 1mL 50% isopropanol to each tube, incubate for 30 minutes at 55°C.
- Remove supernatant, and repeat washing with isopropanol/water until gel pieces are blank. Remove solution.
- Add 100µL 100 % isopropanol to each tube. Incubate at room temperature for 15 minutes until the gel pieces become white. Remove the solution.
- Add 100µL DTT solution to all tubes and incubate for 1 hour at 56°C.
- Remove solution, add 100µL iodacetamide solution and incubate in dark for 1 hour.
- Remove solution and wash three times with 100µL acetonitrile.
- Add 30µL 16ng/µL trypsin solution, or enough for covering the gel pieces. Incubate for 30 minutes (until the gel pieces are blank) on ice.
- Remove trypsin solution and add 100µL [50mM] ammoniumbicarbonate, or enough to cover the gel pieces. Incubate overnight at 37°C
- Add 30µL 5% formic acid, and 30µL 100% acetonitrile to each tube. Incubate at 37°C for 20 minutes.
- Transfer supernatant (containing peptides) to a new microfuge tube. Extract peptides from gel pieces one more time with 30µL 5% formic acid, and 30µL 100% acetonitrile.
- Extract remaining gel pieces with 60µL acetonitrile, by incubating at 37°C for 20 minutes.

-Transfer solution to the new microfuge tube and dry the solution in a speedvac.

MS Analysis

Nanoflow On-line Liquid Chromatographic MS Analysis of Proteolytic Peptides received from Anders Moen, UiO:

Reverse phase (C18) nano online liquid chromatographic MS/MS analyses of proteolytic peptides were performed using a HPLC system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with corresponding autosampler, column heater and integrated switching valve. This LC system was coupled via a nanoelectrospray ion source to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For the analyses, 5 μ l of peptide solution was injected onto the 5 x 0.3-mm extraction column filled with Zorbax 300 SB-C18 of 5 μ m particle size (Agilent). Samples were washed with a mobile phase of 97 %/ 0.1% formic acid/3 % acetonitrile. The flow rate was 10 μ l/min provided by the capillary pump. After 7 min, the switching valve of the integrated switching valve was activated, and the peptides were eluted in the back-flush mode from the extraction column onto the 150 x 0.075-mm C18, 3 μ m resin, column (GlycproSIL C18–80Å, Glycpromass, Stove, Germany). The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1% formic acid. Chromatographic separation was achieved using a binary gradient from 10 to 55% of acetonitrile in 60 or 210 min. The flow rate was 0.2 μ l min⁻¹ provided by the nanoflow pump. Mass spectra were acquired in the positive ion mode applying a data-dependent automatic switch between survey scan and tandem mass spectra (MS/MS) acquisition. Peptide samples were analyzed with a collision induced dissociation (CID) fragmentation method, acquiring one Orbitrap survey scan in the mass range of m/z 200–2000 followed by MS/MS of the six most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for survey scan at a resolution of 30,000 at m/z 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180s.

Data Analysis

Data Analysis received from Anders Moen, UiO:

Mass spectrometric data were analyzed with a proteomdiscoverer search against an RppH-6xHN/5'RppH-GFP-6xHN protein sequence database. The mass tolerances of a fragment ion and a parent ion were set to 0.5Da and 10ppm, respectively. A false discovery rate of 0.01 was required for proteins and peptides with a minimum length of 6 amino acids.

3.5.7 Protein activity assay

3.5.7.1 *In vitro* synthesis of RNA

RNA synthesis

Solutions from a MEGAscript[®]Kit (Ambion[®]) were used for RNA *in vitro* synthesis. The template used was a Sk+_mod_nudix vector (see section 3.2.1) containing a T7 RNA polymerase promoter site.

Procedure:

-Linearize your DNA template of choice by restriction cutting. My template was cut with SmaI, which cut in the middle of the *rppH* Nudix gene. The cutting provides a stop site for the polymerase.

- Confirm the cutting by examine 1 μ L of the template on an agarose gel.
- Terminate the restriction digest by adding 1/20th volume of EDTA [0.5M], 1/10th volume of sodium acetate [3M] and two volumes of ethanol [96%].
- Thaw and assemble the reagents in the written order in room temperature: DEPC water, 2 μ L ATP solution, 2 μ L CTP solution, 2 μ L GTP solution, 2 μ L UTP solution, 2 μ L 10X reaction buffer, 1 μ g linear template DNA and 2 μ L enzyme mix. Amount of DEPC water is adjusted so that the total volume is 20 μ L. Mix gently by pipetting.
- Incubate reaction at 37°C for 2-4 hours. My reagent was incubated for 6 hours since longer incubation time (up to 16 hours) is recommended for transcripts less than 500bp.
- Add 1 μ L TURBO DNase, mix and incubate at 37°C for 15 minutes to remove template DNA.

5% polyacrylamide gel containing urea

The synthesized RNA was further checked on a 5% polyacrylamide gel with urea.

Procedure:

- For making a 5% polyacrylamide gel, mix the following reagents in the written order: 2.4g urea, 0.50mL TBE [10X], 0.84mL acrylamide [30%], 2.5 μ L Ethidium bromide [1mg/mL] and 1.8mL DEPC water. Warm up the solution until the urea is dissolved, cool on ice and add 10 μ L TEMED and 12.5 μ L APS [10%]. Pour the solution into assembled glass plates and insert a comb. Wait until the gel is solid.
- Mix 1 μ L of each RNA sample with 4 μ L DEPC water and 5 μ L loading buffer. Denature the samples at 80°C for 3-5minutes.
- Add 1 μ L of 0.1 μ g/ μ L EtBr to the samples.
- Load 10 μ L of each sample and fractionate the samples for 30 minutes at 150V.

3.5.7.2 Purification of transcripts

After confirmation of successful RNA synthesis by polyacrylamide gel electrophoresis, the samples had to be purified. One of the samples, RNA1, was purified with a MEGAclean™ Kit (Ambion®), while the other sample, RNA2 was purified by phenol/chloroform extraction.

Phenol/chloroform extraction:

- Add 115 μ L DEPC water and 15 μ L ammonium acetate stop solution (ammonium acetate [5M], EDTA [100mM]) to the sample. Mix thoroughly.
- Extract with an equal volume of phenol/chloroform followed by extraction with an equal volume of chloroform. Transfer the aqueous phase to a new microcentrifuge tube.
- Add an equal volume of isopropanol for precipitating the RNA. Mix and leave the tube at -20°C for at least 15 minutes.
- Centrifuge for 15 minutes at 4°C at maximum speed. Remove supernatant and wash the pellet with 1mL ethanol [70%].
- Centrifuge for 5 minutes at 4°C at maximum speed. Remove supernatant and dry the pellet in a vacuum centrifuge.
- Resuspend the RNA in 30 μ L DEPC water.

MEGAclear™ Kit (Ambion®):

- Bring the sample to 100µL with Elution Solution. Mix gently.
- Add 350µL of Binding Solution Concentrate to the reaction and mix.
- Add 250µL of ethanol [96%], mix and apply the sample to the Filter Cartridge which is placed into a supplied Collection/Elution tube.
- Centrifuge 15-60 seconds at 10000-15000g (until the mixture has passed through the filter). If subjected to RCFs over 16000g the Filter Cartridges might brake/deposit glass fibers in the sample. Discard the flow through.
- Wash with 2 x 500µL Wash Solution. Centrifuge the tube and discard the flow through as in the previous step after each wash.
- Remove the last traces of Wash Solution by an additional centrifugation for 10-30 seconds.
- Transfer the Filter Cartridge into a new Collection/Elution Tube. Apply 50µL of Elution Solution to the center of the filter, close the cap and heat the tube for 5-10 minutes at 65-70°C.
- Centrifuge the tube at room temperature for 1 minute (RCF 10000-15000g) for recovering the RNA.
- Repeat the two previous steps with a new aliquot of 50µL Elution Solution for maximizing RNA recovery.
- For concentrating the 100µL elute, add 10µL Ammonium acetate [5M] and 275µL ethanol [96%]. Mix and incubate at -20°C for 30 minutes.
- Centrifuge at 4°C for 15 minutes at maximum speed. Remove the supernatant and wash the pellet with ethanol [70%].
- Centrifuge at 4°C for 5 minutes at maximum speed. Remove the supernatant and dry the pellet in a vacuum centrifuge.
- Resuspend the pellet in 30µL DEPC water.

Both solutions were further quantified by UV absorbance ($O.D_{260nm}$), see section 3.4.8.

3.5.7.3 Activity assay and analysis

Four different enzymes were tested, two positive controls *E.coli1* and *E.coli2* and the two enzymes of interest sRppH and RppH-6xHN. *E.coli1*, RppH-6xHN and sRppH were expressed and purified by affinity chromatography from *E. coli* prehand, while *E.coli2* was ordered from New England Biolabs. To verify the assay, a set of standard curves was prepared additionally.

Samples:

Standard curves

- Prepare PPI standards by dissolving different amounts of *tetra*-sodiumdiphosphate x 10 H₂O in DEPC treated dH₂O to a final volume of 40 µL. (Concentrations for the standards used are shown in table 4.3, section 4.7.3.)
- Analysed the samples with a PPI detection kit (Lonza).

Activity assay

- Mix 2µg RNA with 1µL of the different enzymes (with preferred concentration) in 1X buffer 2 (NEB) buffer to a final volume of 40 µL.
- Incubate the samples at 37°C for 30 minutes.

-Analyse the samples with a PPI detection kit (Lonza). (If noted, the sample was denatured by 5 minutes boiling before conversion and detection.)

Detection:

To detect inorganic pyrophosphate a PPILight™ inorganic pyrophosphate assay (Lonza) was used. The produced pyrophosphate is first converted to ATP. Further luciferase produces light from the newly produced ATP. The amount of light is directly proportional to the amount of PPI present in the sample.

Procedure:

-Reconstitute reagents and allow to equilibrate to room temperature as described in the manual instructions.

-Prepare a negative control well, final volume 40µL.

-Add 20µL PPILight™ Converting Reagent to each sample and incubate at room temperature for 30 minutes.

-Add 20µL PPILight™ Detection Reagent to each sample and incubate at room temperature for 30 minutes.

-Read the luminescence (0.1s integrated reading) in an Illuminometer.

4 RESULTS

Prior to the start of this project, a RNA pyrophosphohydrolase was identified in *E. coli* (Deana et al. 2008). Suspecting that a RNA pyrophosphohydrolase homologue was present in *C. reinhardtii*, a BLAST search against the *C. reinhardtii* genome (www.phytozome.net) was performed with the *E. coli* RppH protein sequence. The search resulted in eight *C. reinhardtii* Nudix proteins. Of these the protein with most homology to the *E. coli* RppH was chosen for further work.

To acquire the sequence of the chosen putative *rppH* gene, total RNA was isolated from *C. reinhardtii* and used as template in reverse transcription using primers that bind to sequences in the annotated 3' UTR region of the transcripts. The resulting cDNA was amplified twice by PCR. Further the sequence was cut and cloned into a Bluescript II Sk+ vector (Stratagene). In order to add a histidine-tag to the protein, the putative *rppH* gene was cloned into the pEcoliC-term6xHN vector (Clontech). To do this the *rppH* sequence was again amplified by PCR on the bluescript_nudix construct, using a 5' primer containing an NcoI restriction site and a 3' primer containing an XbaI restriction site. The amplification product was cut with NcoI/XbaI and finally ligated into a pEcoliC-term6xHN vector (Clontech), also termed the starting vector (pEcoli_nudix) in this thesis (Figure 4.1).

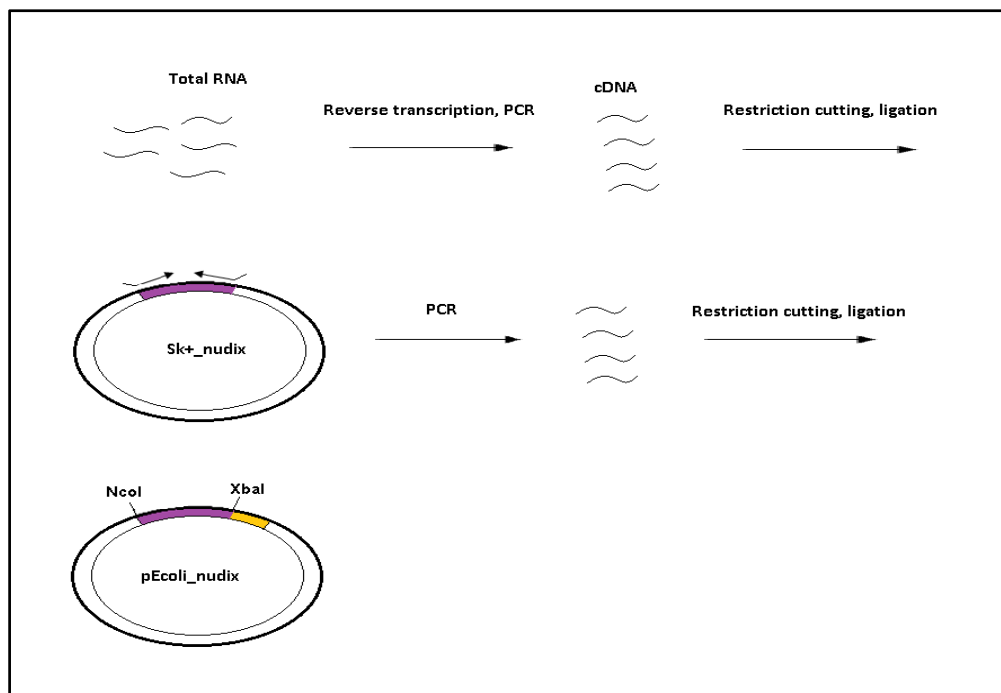


Figure 4.1 Overview of previous cloning. Total RNA was isolated followed by reverse transcription and PCR. The cDNA (purple) was further cloned into a Bluescript II Sk+ vector (Stratagene). PCR with new primers was performed to add NcoI and XbaI restriction sites to the ends of the gene. Finally the fragment was inserted into a pEcoliC-term6xHN vector (Clontech), adding a histidine-tag (yellow) to the C-terminal end of *rppH*.

Our chosen translation start codon is not the same as the translation start codon suggested in the genomic sequence at the phytozome website (www.phytozome.net) for this hypothetical RppH protein. The specific start codon was chosen by Uwe Klein as a result of several elements. First of all the chosen codon is part of a Kozak consensus sequence, which is needed for translation. The length

of the gene compared to other homologous Nudix genes, also became more equal when this optional start codon was used. Both start codons can be seen in Appendix V.

4.1 The *rppH*-6xHN transformation vector

4.1.1 Overview over the transformation vector

The final transformation vector (Figure 4.2), was created by using the pChlamiRNA3int vector as backbone (Molnar et al. 2009). In the construct the RppH protein contains a thrombin cleavage site and a histidine tag at its C-terminal end. The histidine tag can be used to purify the protein and in localization of RppH-6xHN by detecting the histidine-tag with a histidine-tag antibody. Further the gene is flanked 5' by the *Chlamydomonas psaD* promoter and 3' by the *psaD* terminator giving the necessary transcription elements. *PsaD* is a nuclear encoded gene and codes for a protein found in the photosystem I complex in the chloroplast. The *psaD* gene is highly expressed and it is assumed that the high expression elements are found in the 5' and 3' regions, since the *psaD* gene does not contain introns (Fischer and Rochaix 2001).

The vector also contains a chimeric *HSP70A-RBCS2* promoter::*aphVIII*::*RBCS2* terminator sequence, providing paromomycin resistance. The *HSP70A-RBCS2* promoter is a strong heat shock promoter securing sufficient expression of the *aphVIII* gene (Wu et al. 2008).

Originally the pChlamiRNA3int vector also contains an *rbcS2* intron however, this intron is lost in the third cloning step (chapter 4.1.2.). It has been shown that by including the *rbcS2* intron in constructs used for ectopic expression in *C. reinhardtii*, transgene expression increase significantly (Lumbreras et al. 1998).

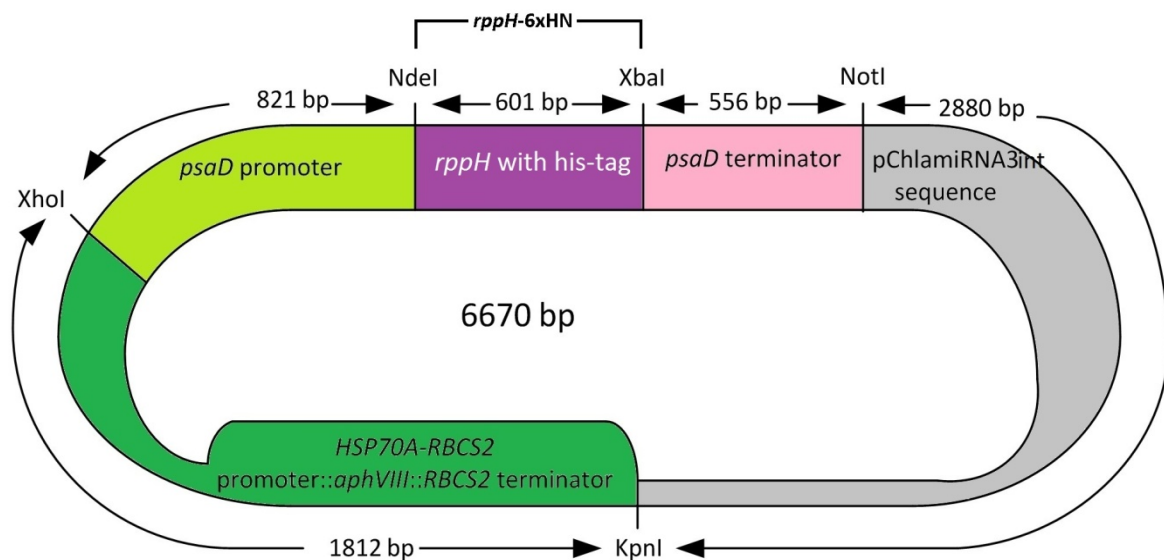


Figure 4.2 The *Chlamydomonas* transformation vector. The *rppH* gene including the histidine tag shown in purple is flanked by the *psaD* promoter (light green) and the *psaD* terminator (pink). Shown in grey is the pChlamiRNA3int backbone sequence while the chimeric *HSP70A-RBCS2* promoter::*aphVIII*::*RBCS2* terminator region is colored emerald green. Restriction sites divide the construct in the colored fragments of the given sizes. The different regions are not drawn to scale.

4.1.2 Subcloning

Subcloning of the *Chlamydomonas rppH* gene was, as shown (Figure 4.3), performed in three main steps, marked A, B and C below.

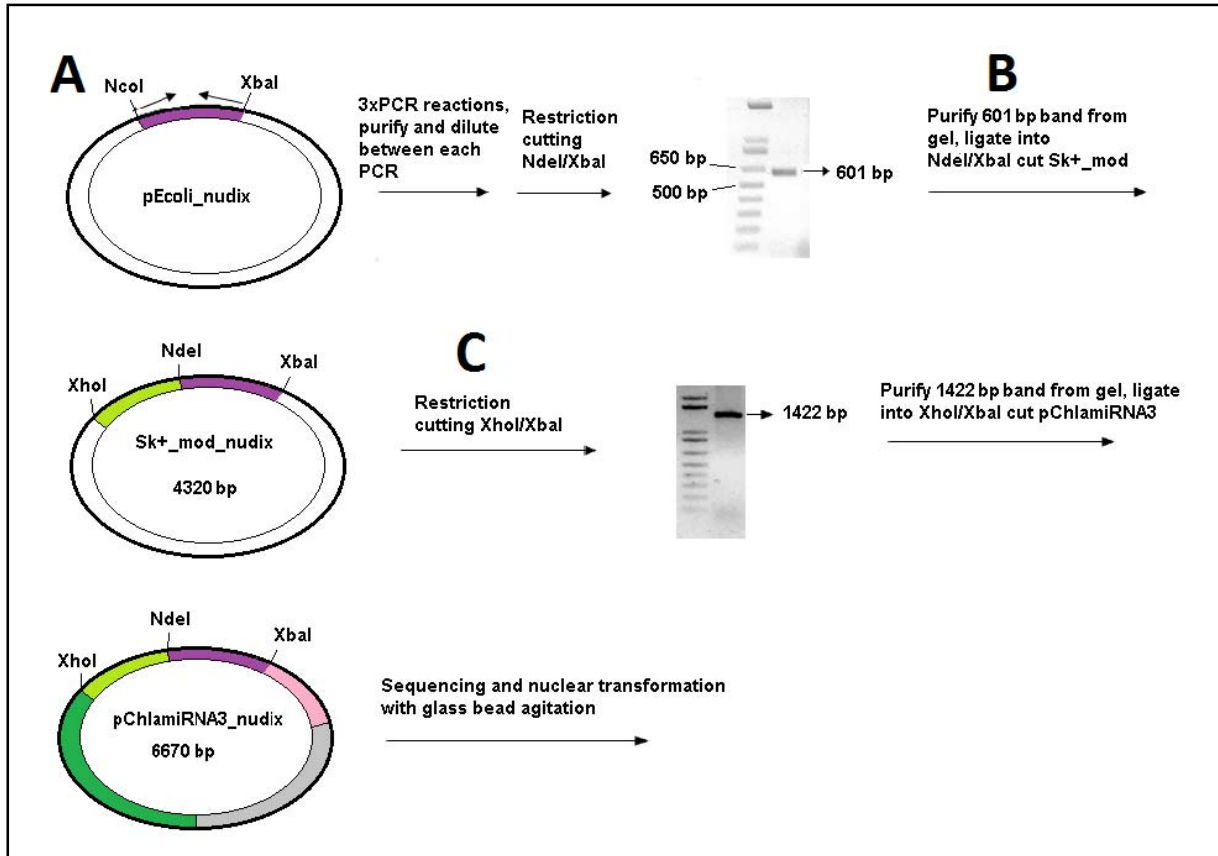


Figure 4.3 Subcloning of *rppH*. A: The *rppH*-6xHN sequence (purple) was amplified from the pEcoli_nudix vector. In this process an NcoI restriction site containing the translation start codon, was converted to an NdeI restriction site. B: The *rppH*-6xHN product was inserted into the intermediate Sk+_mod vector (containing the 5'psaD promoter region, green). C: 5'psaD-*rppH*-6xHN was cloned into the final transformation vector pChlamiRNA3int, which contains the 3'psaD terminator sequence (pink), the *aphVIII* region (emerald green), and backbone sequence (gray).

A:

First three PCR-reactions were performed on a pEcoli-Cterm6xHN vector (Clontech) containing the sequence of *rppH*, from now on called pEcoli_nudix. The primers used amplified the *rppH* gene, which had been inserted between the restriction sites NcoI and XbaI (performed by Uwe Klein and Maria L. Salvador). The PCR reactions were all done using the same 3' primer attaching at the XbaI restriction site, but with different 5' primers all attaching at the NcoI restriction site. Each 5' primer differed in one base, resulting in the change of an ACC triplet to CAT, and conversion of an NcoI restriction site to an NdeI restriction site. Between each PCR the PCR amplification product was fractionated on an agarose gel and purified. Restriction cutting NdeI/XbaI was then performed on the PCR product, resulting in a 601 bp DNA fragment. The DNA was analysed and purified on an agarose gel.

B:

Second, the DNA was ligated into an NdeI/XbaI cut Sk+_mod vector. This Bluescript II Sk+ vector had previously been modified (thereof the name Sk+_mod) by insertion of the XhoI-XbaI sequence from pChlamiRNA3int (performed by Uwe Klein). The XhoI-XbaI sequence contains the *psaD* promoter region used for expressing the cloned gene, an *rbcS2* intron, and two NdeI restriction sites in addition to other sequences. Since the Sk+_mod vector was cut with NdeI/XbaI, cutting the vector three times, the intron sequence was lost with the release of two fragments on 251 bp and 161 bp. The *rppH* gene with a sequence coding for a thrombin cleavage site and a histidine tag at its C-terminal end was ligated into the vector directly downstream of the *psaD* promoter (Figure 4.4).

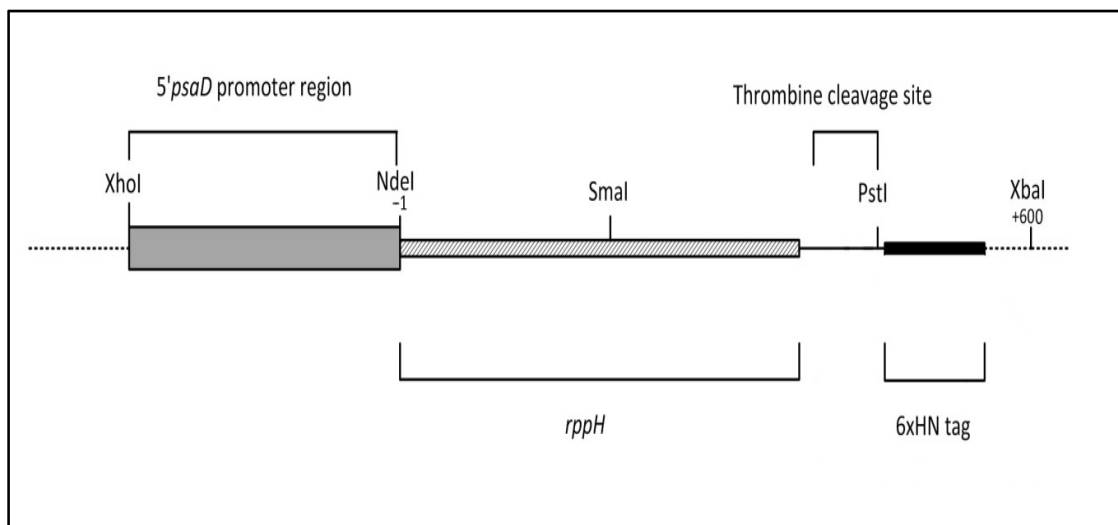


Figure 4.4 The Sk+_mod_nudix construct. *RppH*-6xHN was ligated into Sk+_mod using NdeI and XbaI restriction sites as shown. This intermediate step placed the *psaD* promoter (grey) upstream of *rppH* (diagonal stripes). The histidine tag is shown in black. Numbers written indicate where the restriction enzymes used for subcloning and verifying the construct cut in the sequence relative to +1, which is the translation start site.

The Sk+_mod_nudix construct was verified by NdeI/XbaI restriction cutting (Figure 4.5). As seen the insert has successfully been integrated in all samples tested. The plasmid DNA was isolated by miniprep from four different colonies. Subcloning was continued with vector DNA from colony number two (lane 3).

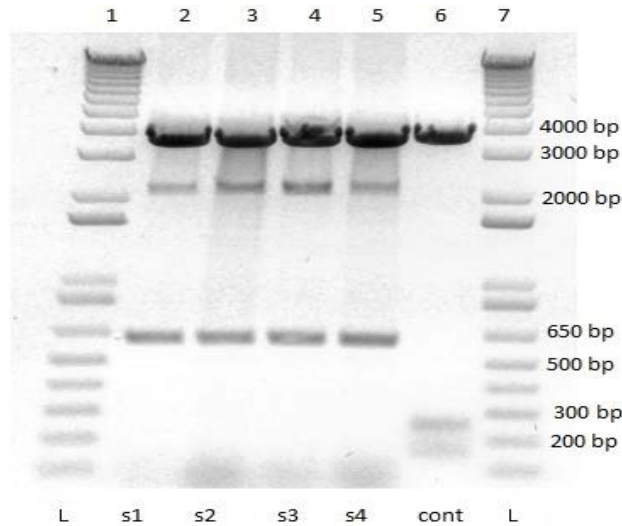


Figure 4.5 Verification of transformants containing the Sk+_{mod}_nudix construct. Plasmid DNA from four different clones were cut with NdeI/XbaI (lane 2-5). A positive control containing Sk+_{mod} plasmid cut with NdeI/XbaI was also included (lane 6). Lane 1 and 7 contain DNA ladders. A band around 600 bp (expected size 601 bp) indicates that the *rppH* gene has been successfully integrated in the vector, which is the case for all the samples tested. Additionally a second band above 2000 bp is seen in sample 1-4, probably representing uncut, supercoiled plasmid. The positive control has as expected no 600 bp band but two lower bands, approximately 150 bp (expected size 161 bp) and 250 bp (expected size 251 bp) in size, corresponding to backbone sequence containing the *rbcS2* intron. All samples including the positive control, has an upper band around 3500 bp (expected size 3719 bp) representing the main part of the vector backbone.

C:

Restriction cutting by XhoI/XbaI was performed on the Sk+_{mod}_nudix construct, releasing a 1422 bp long DNA fragment containing the *rppH*-6xHN and the *psaD* promoter. The band was purified from an agarose gel and ligated into an XhoI/XbaI restriction cut pChlamiRNA3int vector. As shown (Figure 4.6) the fragment was cloned upstream of the *psaD* terminator sequence, completing the construct.

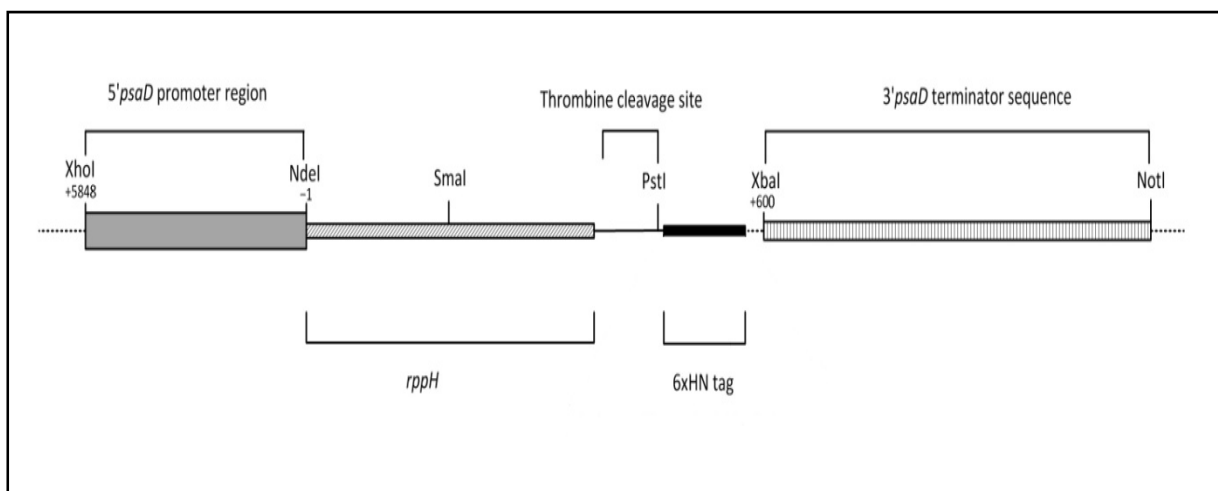


Figure 4.6 The pChlamiRNA3int_nudix construct. The 5'*psaD* promoter region and *rppH*-6xHN was ligated into pChlamiRNA3int using XhoI and XbaI restriction sites as shown. This final step completed the construct by placing the *psaD* terminator (vertical stripes) downstream of *rppH*-6xHN (diagonal stripes). The histidine tag is shown in black and the *psaD* promoter is shown in grey. Numbers written indicate where the restriction enzymes used for subcloning and verifying the construct cut in the sequence relative to +1, which is the translation start site.

The pChlamiRNA3int_nudix construct was verified by XhoI/XbaI restriction cutting (Figure 4.7) and sequencing. As seen from Figure 4.7 all the colonies tested harbored the pChlamiRNA3int_nudix vector. Colony number one was chosen for further work. Nuclear transformation of *C. reinhardtii* with the pChlamiRNA3int_nudix vector was performed by glass bead agitation.

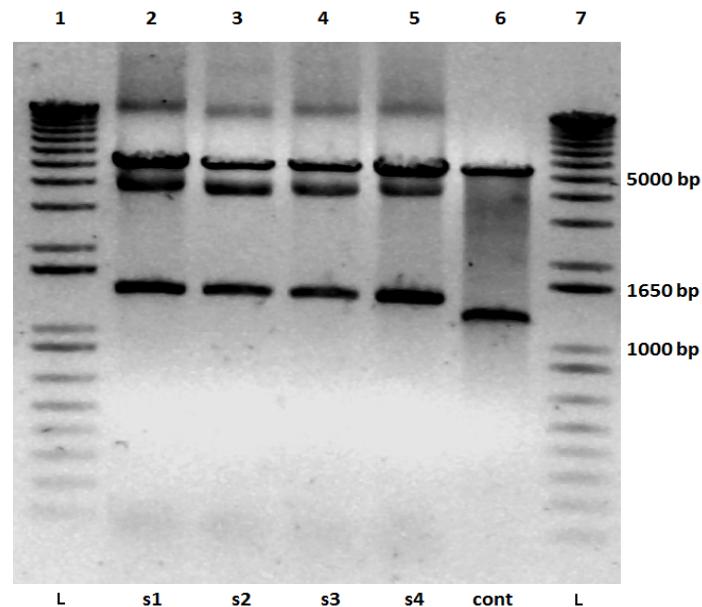


Figure 4.7 Verification of transformants containing the pChlamiRNA3int_nudix construct. Four miniprep samples with plasmid DNA from four different clones (lane 2-5) were checked for the pChlamiRNA3int_nudix construct (lane 2-5) by XhoI/XbaI restriction cutting. A positive control consisting of pChlamiRNA3int vector without insert was also included (lane 6). Lane 1 and 7 contain DNA ladder. A band around 1400 bp (expected size 1422 bp) representing *rppH* and the *psaD* promoter indicates that the sequence successfully has been integrated in the vector. The upper band, between 5000 bp and 6000 bp (expected size 5248 bp) represents the vector backbone. As seen an additional band around 4000 bp is present in sample 1-4, most likely being uncut supercoiled plasmid. For pChlamiRNA3int a band approximately 1200 bp in size (expected size 1233 bp), not containing *rppH*-6xHN, should appear. The Figure shows that all the four colonies contain the pChlamiRNA3int_nudix construct.

4.2 Screening for positive *Chlamydomonas rppH* transformants

4.2.1 PCR

After nuclear transformation by glass bead agitation, the cells were grown on paromomycin plates for selection. The colonies were later transferred to HS paromomycin plates containing a grid, and finally grown in HS medium before total DNA was isolated. For identifying a transformant capable of high expression of RppH-6xHN the DNA isolated from transformants was screened by PCR using a number of different primers (for primer sequences see Appendix III). Eighteen transformants were screened in the first PCR. Three transformants were further checked with controls (Figure 4.9). Two of these were decided upon for a second PCR (Figure 4.10), which resulted in selection of the final transformant, transformant four. Southern blot was further conducted, but after negative results (not shown) new PCRs were performed for additional checking transformant number four and to screen for new transformants (Figure 4.11). Finally a PCR with paromomycin primers was done (Figure 4.12). The different primers used and the sequences they anneal to, are shown in Figure 4.8.

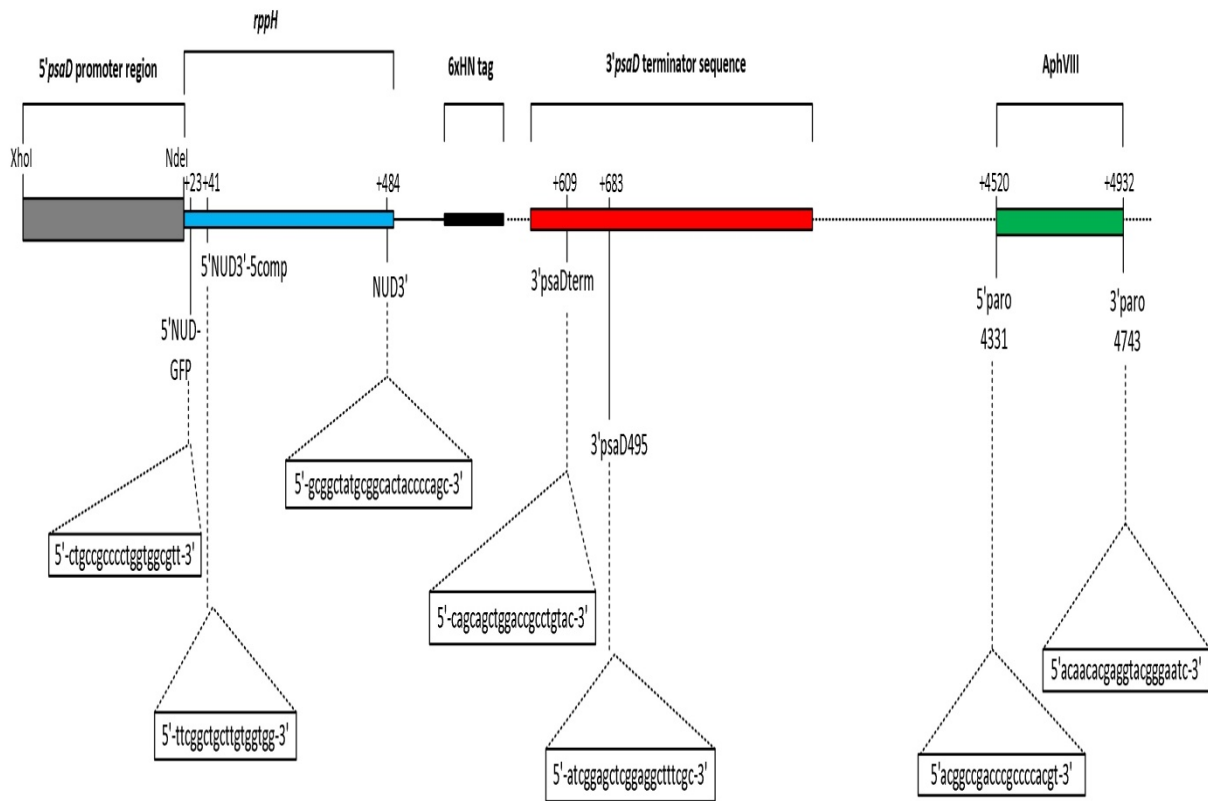


Figure 4.8 Map of the transformation vector including primer binding sites. The map shows a part of the transformation vector, the primers used in screening and the sequences which they anneal to. The numbers indicate the 5' end nucleotide which the primers anneal to relative to +1, which is the translation start site. The 5' *psaD* promoter region is shown in gray, the *rppH* region is shown in blue, while the 3' *psaD* terminator sequence is represented with red. The histidine tag (black) and the *aphVIII* region (green) can also be seen. The map is not drawn to scale.

The primers used in the first PCR are called 5' NUD3'-5comp and 3' *psaD*term (primerset 1). These primers are expected to amplify a 589 bp sequence if the transformation vector has been incorporated as expected. As seen from Figure 4.9, sample 3 and sample 4 both have a strong band which is between 500 bp and 650 bp in length. This is probably the correct band corresponding to most of the *rppH* sequence and the 5' end of the *psaD* terminator sequence. There are however additional bands, which is not uncommon when performing PCR on genomic DNA. Most of the unspecific bands found in the samples can also be seen in the negative control sample wtDNA, indicating that the sequences amplified are not specific for the transformants but probably present in the cw15 wild type. The 3' end primer is a *psaD* primer, attaching to the 3' UTR of the *psaD* gene. *PsaD* is present in *C. reinhardtii* wildtype, and therefore might amplify additional bands. No band with the correct size was amplified for sample 30, and the sample was therefore discarded. Sample 3 and 4 were chosen for additional analysis.

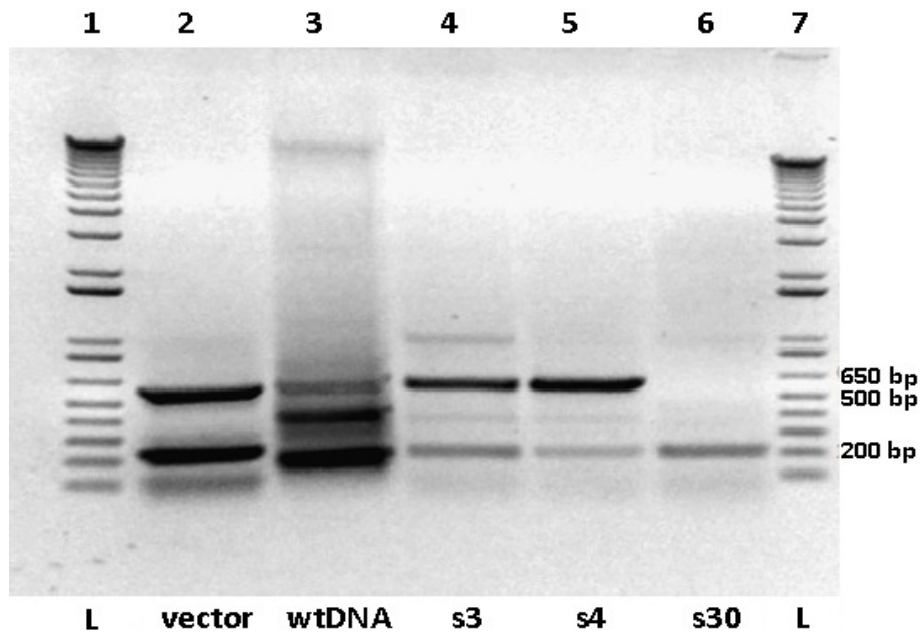


Figure 4.9 PCR using primerset 1. Amplification products from a PCR performed with primerset 1 using template DNA from three different samples (colony 3, 4, 30), cw15 wtDNA, and the final transformation vector. The transformation vector (lane 2) represents a positive control, while the wtDNA (lane 3) represents a negative control. Amplification of a band around 600 bp (expected size 589 bp) indicates a successful transformation. As seen among the amplification products for colony 3 (lane 4) and 4 (lane 5) such a band is present on line with the positive control. There are some additional bands in the controls, which are also present in the samples. These bands are a result of unspecific binding, which could be decreased by increasing the annealing temperature. Because one of the unspecific bands in the negative control is close in size to the 600 bp band in the positive control, the samples have to be further validated.

In the next PCR only *rppH* primers were used (primerset 2). In this way we could check both if the transformants successfully had incorporated the exogenous intron-free *rppH* gene and compare its size with the intron-containing endogenous *rppH* gene. The expected size of the amplified sequence from the exogenous gene is 467 bp. Since the endogenous gene contains introns, a sequence of approximately 1500 bp (expected size 1541 bp) should be amplified. Details of the endogenous gene sequence can be found in Appendix V. The PCR is also performed on the transformation vector, a control which should result in amplification of exogenous *rppH*, and on cw15 wtDNA, another control expected to amplify endogenous *rppH*. As seen in Figure 4.10 two products with the correct size were amplified in sample 4 (lane 5). Transformant number four was therefore chosen for Southern analysis. However due to negative results from Southern analysis, the 467 bp amplification product (assumed to result from the exogenous gene sequence) from sample number four was later verified by sequencing.

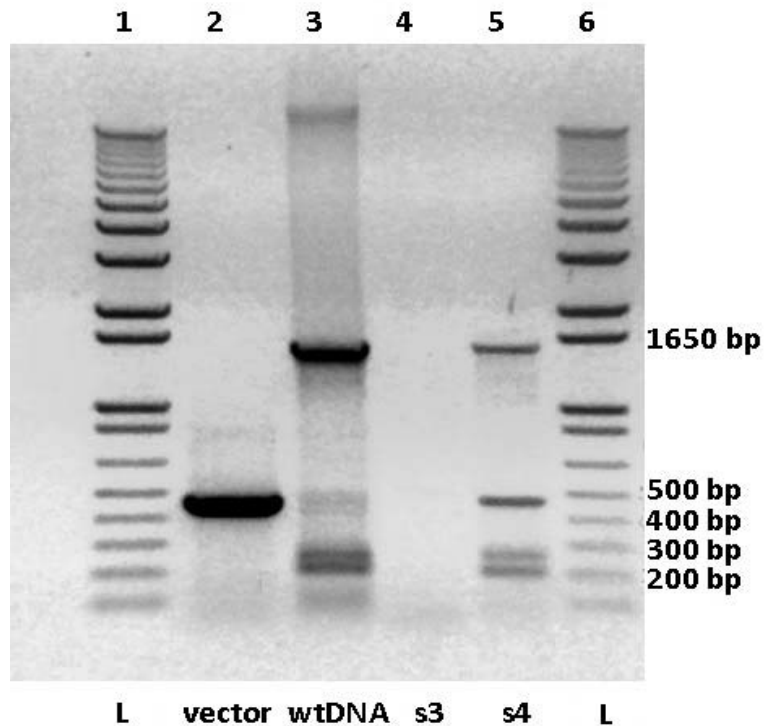


Figure 4.10 PCR using primerset 2. PCR reactions were performed on the transformation vector (lane 2), cw15 wtDNA (lane 3), colony 3 (lane 4) and colony 4 (lane 5), since colony 3 and 4 had positive results in the first PCR. As seen a positive result was only seen for colony 4. Amplification from the transformation vector resulted in one band around 450 bp as expected, representing the exogenous gene. Regarding the amplification products from wtDNA it consists of one strong band around 1500 bp (expected size 1541 bp) representing the endogenous gene and two lower bands representing unspecific binding which is also amplified in colony four. It is a bit difficult to amplify sequences over 1000 bp from genomic DNA, which could be the reason for why the endogenous gene not is present in colony 3. Another option is that the PCR did not work properly, since the unspecific bands also are lacking. However the controls should then additionally lack amplification products, which is not the case. Human error during pipetting of the reaction mixture components into the different tubes containing the samples is therefore the most likely explanation.

As mentioned the Southern analysis of transformant number four gave negative results and an additional PCR with new primers was therefore performed. Several new colonies were checked and transformant number four was checked one more time. First screening of new colonies was performed, and positive transformants were further checked properly with controls. Primers used in the PCR were named 5'GFP NUD and 3'psaD495 (primerset 3). The 5'GFP NUD primer attaches to the 5' end of the *rppH* gene, while the 3'psaD495 primer attaches to the 3'psaD terminator sequence. An amplification using these two primers should give a band of 680 bp if the transformants contain the insert. As seen in Figure 4.11, the PCR result was positive for all samples.

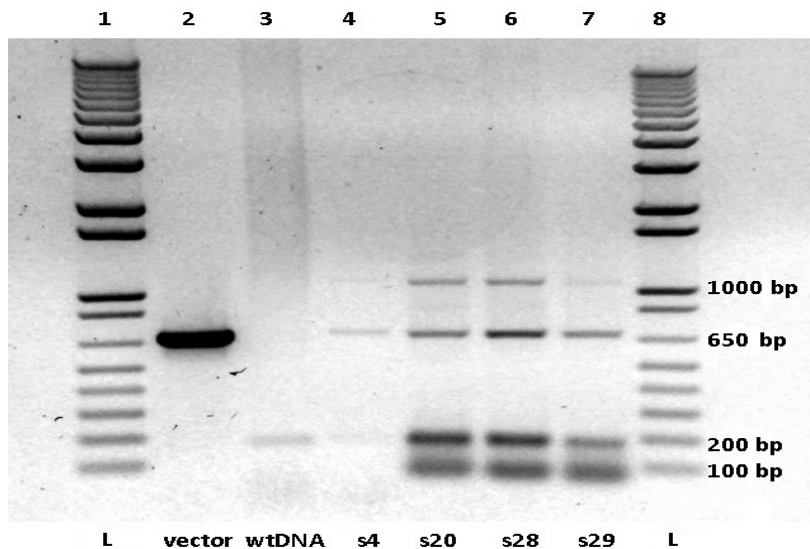


Figure 4.11 PCR using primerset 3. All samples (lane 4-7) contain a band above 650 bp equal in size to the band amplified from the positive control (lane 2), indicating that they all contain the exogenous *rppH* gene. However the amplification products in sample 4 are very weak. This might be due to old template DNA used in the reaction. The negative control (lane 3) only contains a single weak lower band which also is present in the samples. Additional bands resulting from unspecific binding can also be seen in the samples, one above 1000 bp and one around 100 bp.

Since the previous PCR gave four samples with a positive result, a last PCR was performed to see if any of the transformants could be ruled out. The primers used were named 5'paro4331 and 3'paro4743 (primerset 4), and amplification of a band of 433 bp was expected. Both primers attach to the *aphVIII* gene, and amplification therefore confirms that the transformants carry the paromomycin resistance gene. Since the colonies were grown on paromomycin plates, *aphVIII* is a gene that all of the transformants should harbor. As seen in Figure 4.12, a positive result was achieved for all samples.

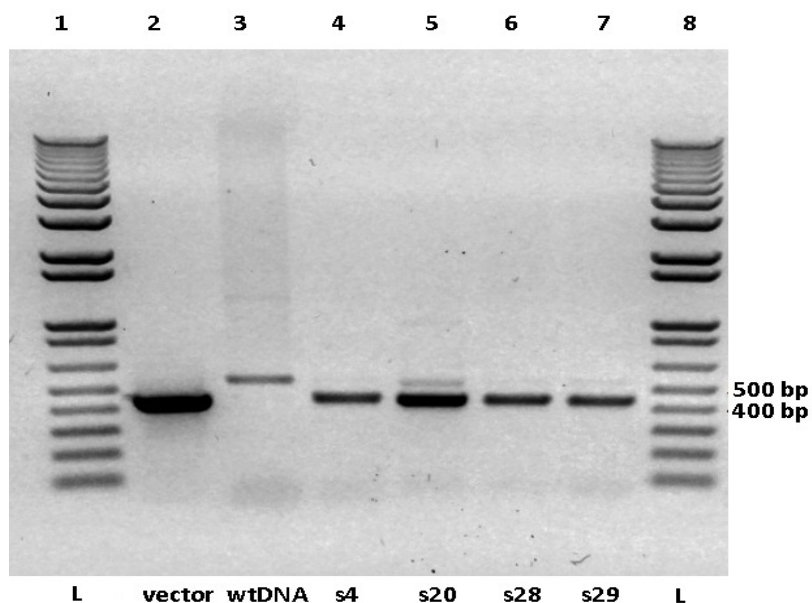


Figure 4.12 PCR using primerset 4. All samples (lane 4-7) contain a band above 400 bp (expected size 433 bp), which also is represented in the positive control (lane 2), indicating that the transformants contain the *aphVIII* gene. In addition they contain a band which is over 500 bp. This band is also present in the negative control (wild-type, lane 3) and is therefore most likely an unspecific amplification product.

The screening resulted in three new transformants possible to use in further analyses, however only sample 28 was chosen for further Southern blot analysis. In addition Southern blot analysis with sample number four was repeated.

4.2.2 Paromomycin resistance

New Southern blots were performed, however again without any positive results (not shown). The samples could have been false positives, if it had not been for the sequencing result from a PCR amplification product for colony number four (using primerset 2), confirming that the product indeed had the correct sequence. Both PCR and Southern blot have pros and cons, and one of the pros using PCR is its sensitivity. Since the PCR results for transformant four were positive while the Southern blot only gave negative results, we started to believe that the cells which should have been pure, in reality were a mixture of transformed and untransformed cells. The amount of DNA from the positive transformants compared to the amount of DNA from untransformed cells could be too small, making detection of the exogenous *rppH* gene with Southern blot difficult.

In theory this should of course not be the case, since the cells are grown on paromomycin plates. However cells not resistant towards paromomycin might grow together with resistant cells and in this way contaminate our sample. When the colonies later are picked and grown in HS liquid media, the nonresistant cells may outgrow the transformed cells. To purify the cells we wanted to try to grow the transformed cells in HS liquid medium containing paromomycin. Previously this has not been possible due to what we think is fast diffusion of the paromomycin into the cells. Paromomycin inhibits protein synthesis in the chloroplast by binding to 70 S ribosomes. Our transformation vector was incorporated in the nuclear genome, and transformed cells resistant to paromomycin will modify paromomycin in the cytosol before the molecule enters the chloroplast. This works on the HS plates where diffusion is lower than in liquid media. In liquid medium the paromomycin might enter the chloroplast before modification in the cytosol has taken place, eventually resulting in cell death. An experiment was therefore set up to see if the cells were able to grow with low concentrations of paromomycin in liquid HS medium.

Transformant number four and cw15 wild type were grown in 0-3.2 µg/ml paromomycin (0-5µL paromomycin [60µg/µL], as marked on the Erlen-meyer flasks containing 75mL HS medium in Figure 4.13). About 1 million cells in liquid HS medium were added to each flask. As seen in Figure 4.13 both cell lines seem to have the same growth in 0µg/mL and 0.4µg/mL paromomycin. However while the transformed paromomycin resistant cells almost grow equally well in 0.8µg/mL paromomycin, the growth is clearly reduced for cw15 wild type cells. This amount of paromomycin could therefore be used for growing transformants. It will not kill untransformed cells but give the transformed cells an advantage, and hopefully the degree of contaminating cells will decrease. For pure cell lines additional work needs to be done.

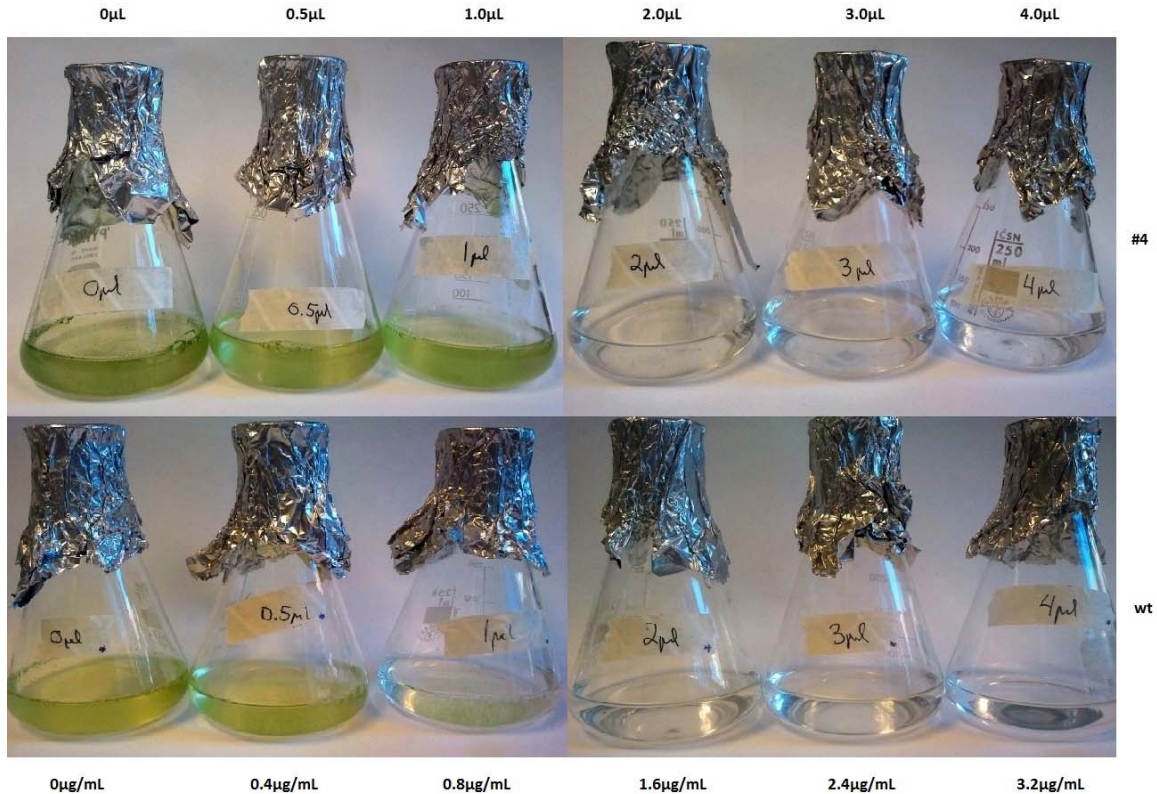


Figure 4.13 Transformant 4 (#4) and cw15 (wt) grown with paromomycin. The top row represents transformant number 4 (#4) while the lower row represents cw15 wild-type (wt). The volume of added paromomycin [60µg/µl] is written above the picture, while the corresponding final concentration is written below. As seen the growth of wild type cells is reduced but still possible in 0.8µg/mL paromomycin, while the growth of transformant #4 not is severely affected. None of the cell lines is capable of growing in more than 0.8µg/mL paromomycin. The pictures were taken 10 days after the start of the experiment.

4.2.3 Northern analysis

Of 50 starting colonies picked from the first paromomycin containing HS plate, transformant number four was the only one with positive results in four different PCR reactions. Expression of the *rppH* construct in transformant four was therefore investigated at the transcript level by Northern analysis.

The cell-line was as explained purified by growth in HS medium containing 0.8µg/mL paromomycin. In addition cells from this culture were collected and spread on a paromomycin containing HS plate. A few visible colonies were seen after three weeks. The colonies were one by one streaked out on a new HS plate containing paromomycin. Furthermore the colony with best growth on the last plate was chosen for Northern analysis. The colony was grown in HS liquid medium and RNA was isolated twice, at the end of the dark cycle and in the beginning of the light, as described in materials and methods. Two controls consisting of RNA isolated from wild type cw15 in light and dark were included in the analysis (Figure 4.14).

As seen in Figure 4.14, a weak band is present in the two RNA samples from *C. reinhardtii* transformant four corresponding to a band that is also present in the controls. The recognized RNA

are probably the endogenous *rppH* transcripts. An additional band of unknown size (the length of the 5' and 3' UTRs in the transcripts of the exogenous *rppH* gene are not known) was expected to appear in the samples isolated from the transformant representing *rppH*-6xHN. The lack of an additional band might be due to low transcription of *rppH*-6xHN. Alternatively the gene might not be expressed at all, or the transcript could have approximately the same size as the endogenous *rppH*, thus being invisible as a separate band. The endogenous *rppH* seems to be transcribed differently in dark and light, however the difference does not seem to be equally large in the two samples, and no conclusion except for that the transcript level of the gene is generally low, can be made. What can be concluded is that the transformant needs further validation at the RNA level.

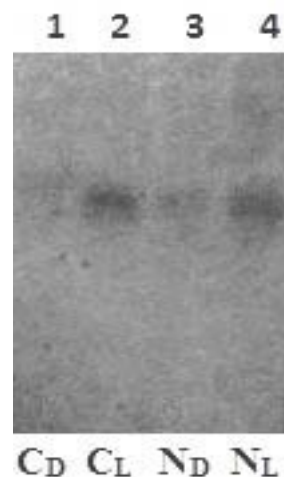


Figure 4.14 Northern blot analysis of the *C. reinhardtii* *rppH*-6xHN transformant 4. Four samples are shown, two RNA samples isolated from wild-type cw15 in light (C_L) and dark (C_D), and two RNA samples isolated from the *rppH*-6xHN transformant four in light (N_L) and dark (N_D). The blot was hybridized to the random primer labeled sequence of the *Chlamydomonas rppH* cDNA. Only the endogenous *rppH* seems to be recognized by the probe, which could imply that *rppH*-6xHN not is transcribed. From the result it looks like the *rppH* transcription level is higher in the light, compared to the transcription level in the dark. However due to lower RNA level in lane 2 compared to the other samples, no conclusion can be made. As seen, the signal from the endogenous *rppH* transcript in the dark-control sample is higher in the gel relative to the other samples, being a result of uneven fractionation of the samples on the agarose gel.

4.2.4 Reverse transcription

In addition to Northern blot analysis (see section 4.2.3) RT-PCR on total RNA from transformant four was performed with the aim to detect *rppH*-6xHN transcripts. The 3'psaD495 primer was used to produce cDNA. As a negative control, reverse transcription was also executed on wtDNA from cw15. Reverse transcription was followed by two PCR reactions to amplify sufficient amounts of the fragments. 3'psaD495 and 5'NUD GFP were used as primers. As positive control we included a sample of the transformation vector in the second PCR reaction. After the first PCR a very weak band around 650 bp (expected size 680 bp) could be seen in the sample representing transformant four. The area containing the band was cut out, purified and an additional PCR was performed. Last the PCR products were fractionated on an agarose gel.

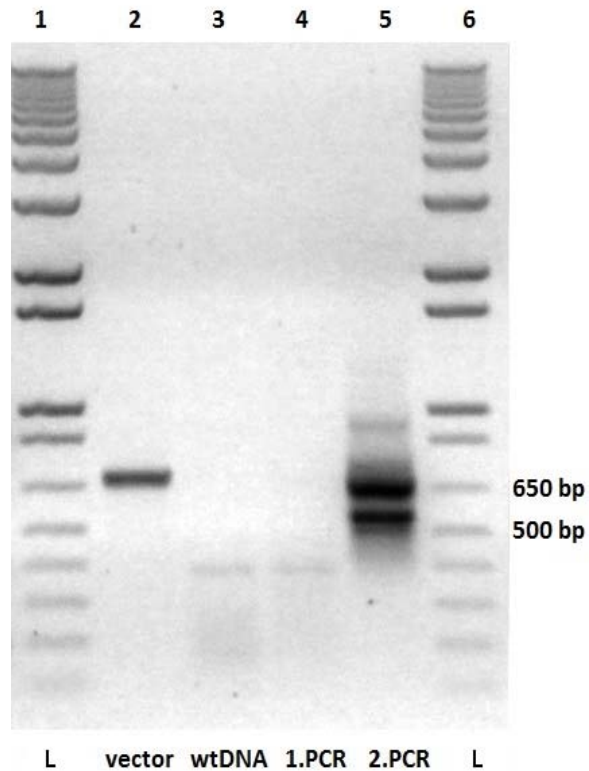


Figure 4.15 RT-PCR. Both the result from the first PCR (lane 4) and the second PCR (lane 5) are shown. In addition a positive control (lane 2) and a negative control (lane 3) are included. DNA ladders are shown in lane 1 and 6. Using the 3'psaD495 primer in the reverse transcription and the 3'psaD495 and 5'NUD GFP primers in the two following PCRs, an amplification product around 650 bp is expected. As seen in lane 5, sample four contains such a band which is comparable in size to the amplification product in the positive control (lane 2). The sample also contains an additional band between 500-650 bp. This could be amplification products from the *psaD* mRNA, since 3'psaD495 is the primer used in the reverse transcription.

To further verify that transformant four transcribes and harbor transcripts of the exogenous *rppH* gene, the PCR amplification products were tested for their ability to hybridize to a probe. A Southern analysis was therefore performed on the second PCR sample. The probe used will anneal to the exogenous *rppH* sequence in addition to some of the *psaD* 3'UTR sequence. It was made by random primer labeling the amplification product from a PCR using primerset 3 and the transformation vector as template. As seen in Figure 4.16 the signal from sample four (lane 2) is quite strong and includes two bands. The strong upper band may represent the amplification product from the exogenous *rppH*. Normally the RNA sample contains some DNA, and several bands are therefore usually resulting doing reverse transcription.



Figure 4.16 Southern analysis on the reverse transcription products. 3 μ L of reverse transcription amplification products from the second PCR on sample four was loaded on an agarose gel (lane 2). As seen two strong bands are visible, most likely corresponding to the two bands shown in the agarose gel (Figure 4.15). The upper band may be the band representing the amplification product from the exogenous *rppH*. The lower band could be a result of amplification of the *psaD* mRNA, which would explain why the probe is hybridizing to the sequence. DNA ladder is seen in lane 1.

4.3 RppH-6xHN protein expression

4.3.1 Expression in *E. coli*

4.3.1.1 SDS-PAGE

Before trying to find the histidine-tagged RppH in *C. reinhardtii*, isolation of RppH-6xHN from *E. coli* was performed. The pEcoli_nudix vector was used as transformation vector. Isolating and separating proteins using affinity chromatography and SDS-PAGE is easier to perform in *E. coli* compared to *C. reinhardtii* since the organism is less complex and contain fewer proteins. However, RppH-6xHN is not codon-optimized for expression in *E. coli*, and the yield was therefore expected to be low.

The pEcoli_nudix vector was transformed into TB1 cells, transformants were picked, grown and a maxiprep was performed as described in materials and methods. Further the purified plasmid was transformed into *E. coli* BL21 protein expression cells. After growth and induction with IPTG, the cells were harvested, sonicated and *C. reinhardtii* RppH-6xHN was isolated using affinity chromatography. The protein was concentrated using an Amicon® Ultra Centrifugal Filters (Millipore) and finally analyzed by SDS-PAGE. RppH-6xHN has a size of 20 637.28 D, and a band around 20 kD is therefore expected. As seen from Figure 4.17 (lane 4) isolation of a protein corresponding to the size of RppH-6xHN, was achieved.

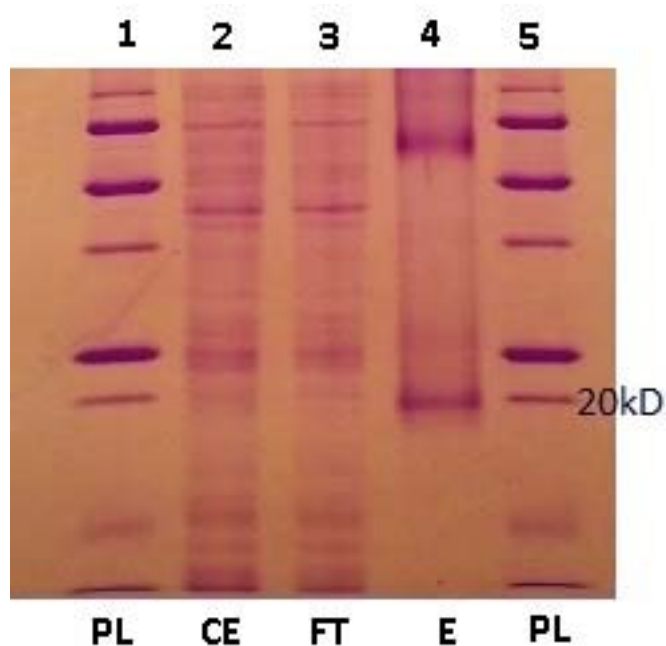


Figure 4.17 *C. reinhardtii* RppH-6xHN isolated from *E. coli* fractionated on a polyacrylamide gel. A 12% acrylamide gel containing protein ladders (lane 1 and 5), crude extract (lane 2), flow through (lane 3), and the concentrated protein (lane 4) is shown. As seen one of the bands in the elute (lane 4) is around 20 kD in size, and the band is probably RppH-6xHN. The lane also contains another strong band that are larger in size. Optimally there should only be one band in this lane since we purified the protein by affinity chromatography.

4.3.1.2 Western blot

Another reason for expressing and isolating RppH-6xHN from *E. coli* was for testing and validating the antibodies we were planning to use. No antibodies towards *Chlamydomonas* RppH exist, but since the protein contains a histidine-tag at its C-terminal end, the original plan was to use a histidine-tag primary antibody together with a secondary peroxidase conjugated antibody, to determine RppH localization. A Luminata™ Forte Western HRP substrate kit (Millipore) was used to detect the antibodies.

SDS-PAGE was performed with the proteins of interest, including the previously antibody-tested histidine-tagged proteins RB38 and RB60 from *C. reinhardtii* as positive controls. The proteins were transferred to a nitrocellulose membrane, and antibodies were added to the membrane as described (3.5.5). A histidine-tag primary antibody (biosite) and a peroxidase conjugated secondary antibody (biosite), called set 1, (see section 3.5.5.2) were used in Figure 4.18. The manufacture claims that the primary antibody should be capable of recognizing C-terminal, N-terminal and incorporated histidine-tags. As seen (Figure 4.18) the antibodies did not recognize the histidine-tag on the *C. reinhardtii* RppH protein (lane 3 and 4). The membrane was stained with Ponceau S after the transfer, and a band in the correct area was seen. We therefore assume that RppH-6xHN was present on the membrane, but for some reason avoided detection. The specificity of the primary histidine-tag antibody was also low. Not only does the antibody bind to proteins in the ladder, it also detects several of the proteins in the flow through. On the other hand, the primary antibody does detect the

positive controls, especially RB38. The signal for RB60 is however not stronger than the unspecific binding of the antibody in the flow through. RB38 contain an N-terminal histidine-tag while RB60 contain a C-terminal histidine-tag.

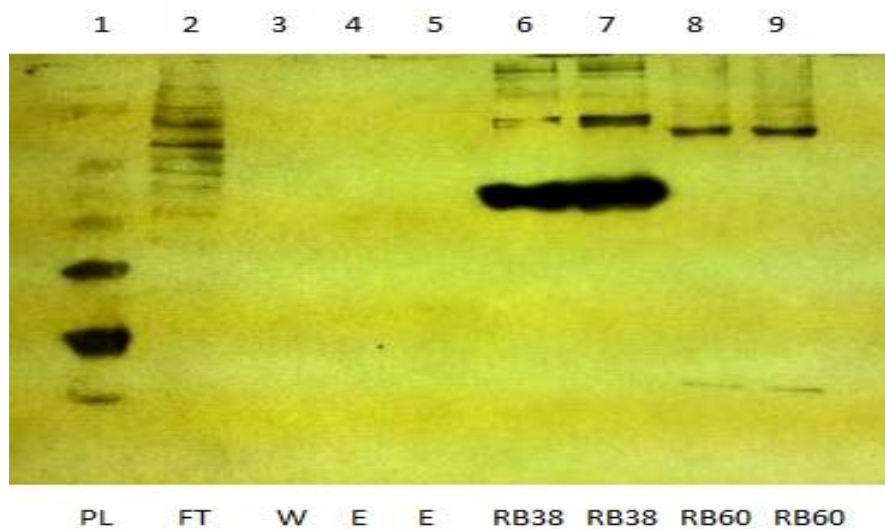


Figure 4.18 Western blot using antibodies from set 1. As seen from the Figure, the histidine-tag primary antibody is not very specific. It detects proteins in the protein ladder (lane 1) and several of the proteins in the flow through (lane 2). Two positive controls RB38 (lane 6 and 7) and RB60 (lane 8 and 9) are present. The antibody does not detect the histidine-tag of RppH found in lane 4 and 5. Lane 3 contains a sample from the wash of the affinity chromatography.

Another reason for the lack of detection could have been that the protein thought to be histidine tagged RppH in the acrylamide gel, was a completely different protein. However, mass spectrometry analysis was performed, verifying the 20 kD protein as RppH-6xHN. Mass spectrometry also confirmed that the C-terminal histidine-tag in the isolated protein was intact.

Since we knew that the correct intact protein was present in the gel, another set of antibodies, set 2 (section 3.5.5.2), were tested. This set only consists of a primary histidine-tag antibody coupled to HRP (Genescript). The antibody in set 2 should as set 1, be able to recognize N-terminal, C-terminal and incorporated histidine-tags according to the manufacture. RB38 was again used as positive control. As seen in Figure 4.19 the new antibody also failed in recognizing the histidine-tag on RppH. The antibody did however recognize several proteins in the protein ladder, which implies that the specificity for this antibody too, is poor. In addition, the signals from the recognized ladder proteins are much stronger than the recognition signal for RB38.

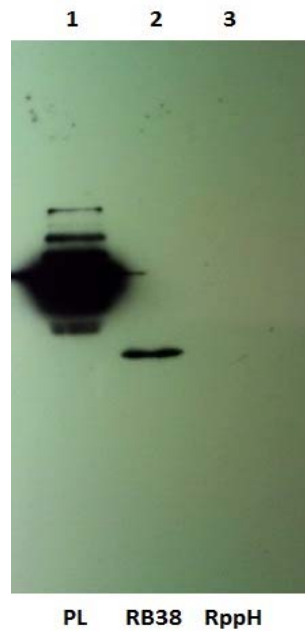


Figure 4.19 Western blot using antibodies from set 2. Lane 1 contains the protein ladder, the RB38 protein which function as a positive control is in lane 2, and the elute containing RppH-6xHN in lane 3. The histidine-tag on RppH was not recognized by the antibody.

We noticed that both antibodies recognized the RB38 protein, which has an N-terminal histidine-tag. RppH-6xHN contains a C-terminal histidine-tag, and we therefore wondered if the result would have been different if the histidine-tag was at the proteins N-terminal end. According to the manufacture both antibodies should recognize the histidine-tag regardless of its position. Nevertheless a new Western blot was performed using a protein from a control vector. The control consisted of the GFP gene cloned into a pEcoli-Nterm6xHN vector (Clontech), adding an N-terminal histidine-tag to GFP. For recognition of the histidine-tagged protein, the primary antibody in set 2 was used. However, once more recognition failed (results not shown).

Since the antibodies were unable to detect RppH-6xHN a new localization strategy involving chloroplast isolation was chosen. By isolating chloroplasts followed by affinity chromatography and SDS-PAGE, comparing the RppH levels in chloroplasts to RppH levels in the total cell should be possible. RppH is most likely not present in very high amounts, and it is uncertain if the protein will be visible on an acrylamide gel. Mass spectrometry of the specific area of the gel is therefore probably necessary.

4.3.2 Expression in *C. reinhardtii*

4.3.2.1 Isolation of chloroplasts from *C. reinhardtii*

Isolation of chloroplasts was performed to compare the amount of histidine-tagged exogenous RppH in chloroplasts with the amount of histidine-tagged exogenous RppH in intact cells. The isolation was done according to the protocol in section 3.4.7. However, as seen from Figure 4.20 the isolation was not successful because the chloroplasts where heavily contaminated with intact cells. The chloroplasts should have a cup-shaped form while intact cells are identified as cells containing a cell membrane and/or flagella. A fraction of isolated chloroplasts were investigated using confocal

microscopy, identifying more intact cells than chloroplasts in the sample. Localization of RppH-6xHN using this procedure was therefore not possible, and other chloroplast isolation protocols should be used. The procedure was tried modified in several ways without improvement.

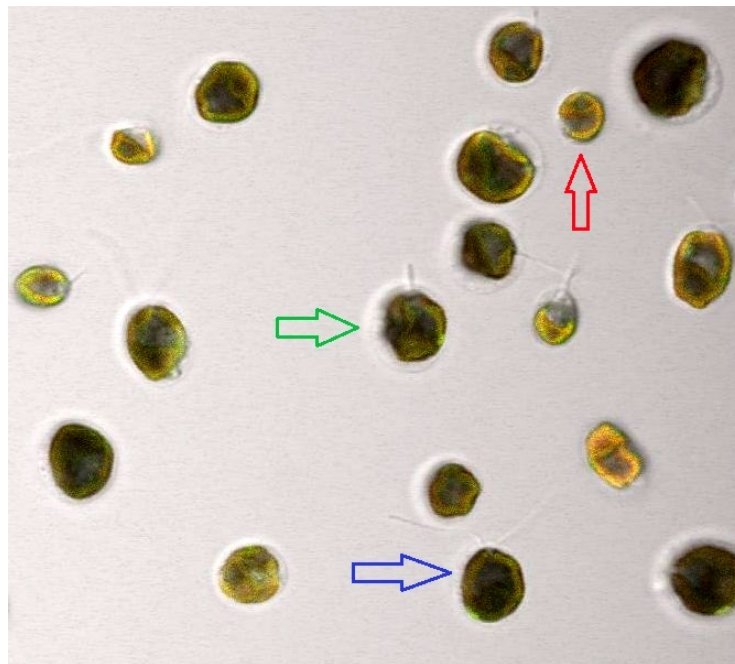


Figure 4.20 Confocal microscopy analysis of isolated chloroplasts. The Figure shows a confocal microscopy picture of what, according to the protocol, should have been isolated chloroplasts. However, the sample consisted mostly of intact cells. Intact cells are recognized by flagella and/or a cell membrane. The green arrow shows a cell having a cell membrane, whereas the blue arrow shows a cell with two clear flagella. A possible chloroplast is indicated by the red arrow.

4.3.2.2 SDS-PAGE

Since we were unable to isolate chloroplasts and the histidine-tag recognition by several antibodies failed, it was not possible to localize RppH-6xHN in *C. reinhardtii* using the original strategy. However proteins were isolated from transformant four to verify the cell-line at the protein level by other means than immunoblotting. Additionally purification of RppH-6xHN from the transformant was tried using affinity chromatography, however the procedure failed (not shown). A crude extract sample from *C. reinhardtii* transformant four was therefore compared to a crude extract sample from wild-type cw15 cells by SDS gel electrophoresis in order to detect the RppH-6xHN protein. RppH-6xHN isolated from *E. coli* by affinity chromatography was used as a control. A weak band, not present in the wild-type sample, was seen in the crude extract of transformant four (Figure 4.21). The band was also equivalent in size to the positive control (purified RppH-6xHN isolated from *E. coli*). With the aim to fully validate the protein as RppH-6xHN the band was cut out and analyzed by mass spectrometry.

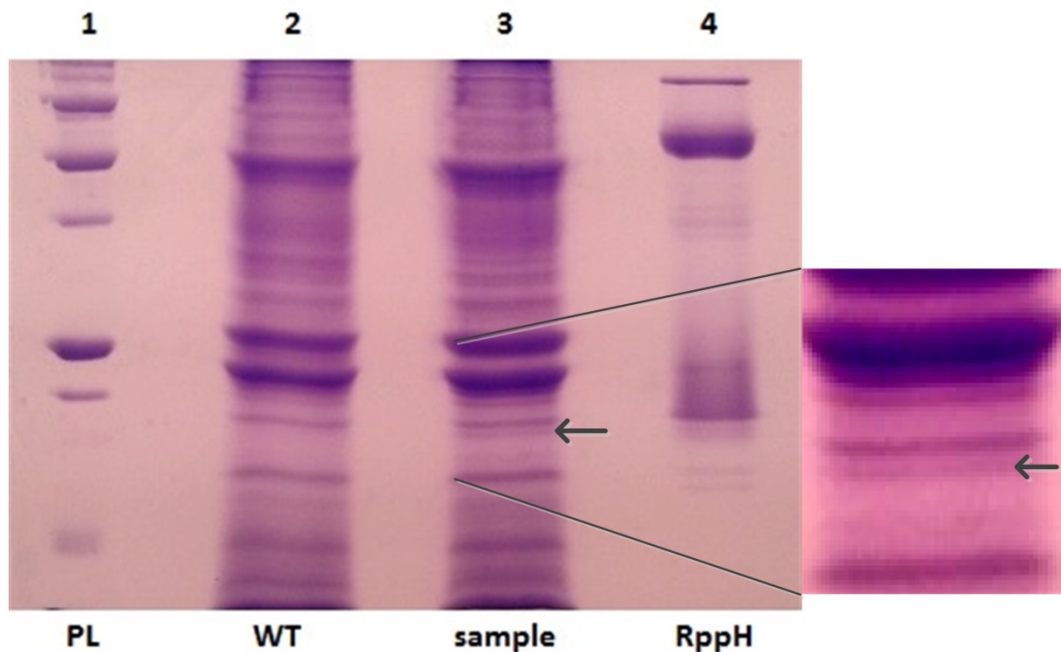


Figure 4.21 SDS-PAGE analysis of *C. reinhardtii* RppH-6xHN transformant four. A 12% acrylamide gel containing a protein ladder (lane 1), crude extract sample from wild-type cw15 (lane 2), crude extract isolated from the RppH-6xHN transformant number 4 (lane 3), and purified RppH-6xHN isolated from *E. coli* (lane 4) is shown. Crude extract from wild-type cw15 is used as a negative control while RppH-6xHN isolated from *E. coli* is used as a positive control. The expected size of RppH-6xHN is around 20 kD, and such a band comparable in size with the positive control is found in lane 3 containing the sample (shown with arrows and magnification). The band is not present in the negative control suggesting the band might represent RppH-6xHN.

4.3.2.3 Mass spectrometry

The area containing the presumed RppH-6xHN was cut out from the gel and digested with trypsin according to the protocol written in section 3.5.6. Mass spectrometry analysis were performed by Anders Moen (UiO) both on the sample (lane 3 containing a crude extract sample from the RppH-6xHN transformant four) and on the positive control (lane 4 containing purified RppH-6xHN isolated from *E. coli*) from the gel shown in the previous section. The amount of RppH peptides detected in the sample was however quite low even though the band which was believed to represent RppH-6xHN in *C. reinhardtii* transformant four is seen quite clearly in the acrylamide gel. This indicates that the band most likely consists of another protein. However, three different RppH peptides were found in the sample, matching verified fractionated peptides in the positive control with an exactness of the m/z -value down to the second decimal (Figure 4.22). Therefore it is almost certain that RppH is present in the crude extract of *C. reinhardtii* transformant four.

A problem is however that no peptide containing the histidine-tag was found in the sample, not surprisingly considering the small amount of RppH-peptides. A possibility is therefore that the positive result derives from the presence of endogenous RppH. Endogenous RppH should not be at the same position as RppH-6xHN in the gel, however human error during the gel cutting might have included a broader area of the gel than necessary. Due to the lack of histidine-tag containing peptides additional analysis should be performed to validate the *C. reinhardtii* RppH-6xHN transformant four at the protein level.

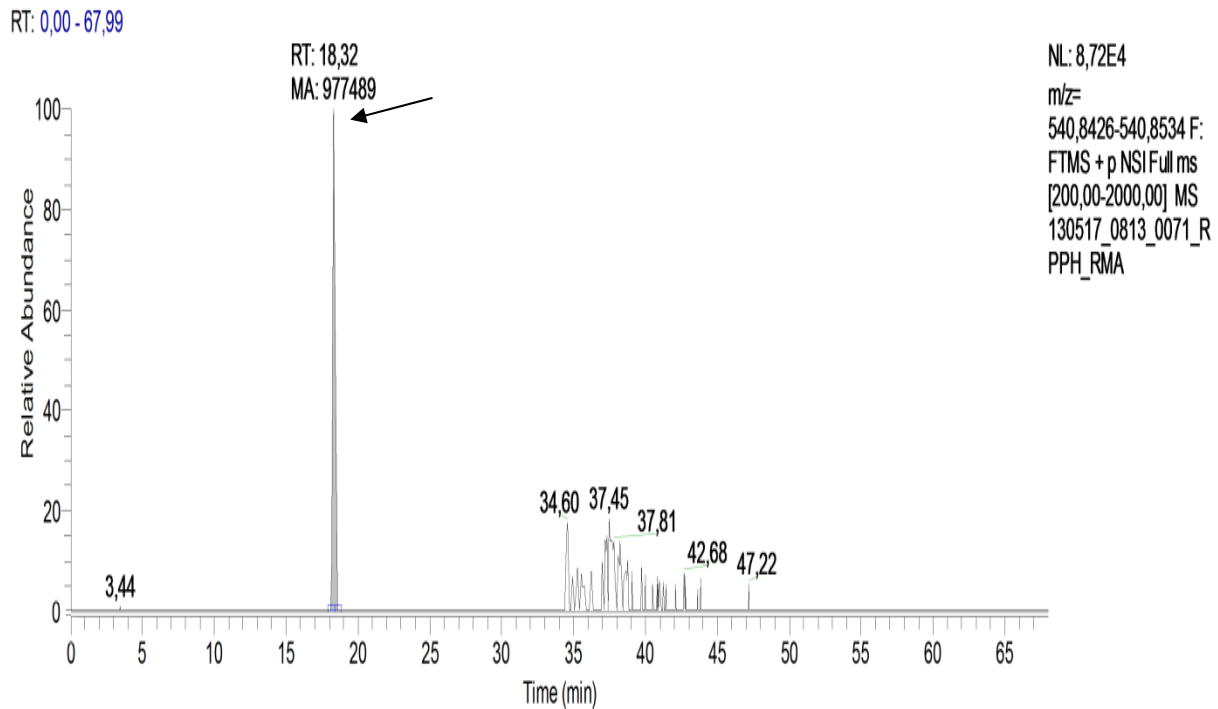


Figure 4.22 Mass spectrum of an RppH-peptide from the *C. reinhardtii* transformant four. Since the content of RppH-peptides in the sample was low, the peptides did not fractionate. However by searching after specific peptides which was seen in the control, three different RppH-peptides were discovered. One of the peptides is shown as a peak in the mass spectrum above, representing the aminoacid sequence VLLAQRPVGK found in RppH-6xHN (arrow). The mass spectrum in the control is compared with the mass spectrum for the sample, verifying that the peptides harbor correct m/z values and is eluted approximately at the same time. By comparing the areal of the peak found in the control (MA; 1411438100) with the areal of the peak found in the sample (MA; 977489), the difference in amount of peptide in the samples becomes clear. For all spectra including the control see Appendix VI. MA represents the areal of the peaks while RT shows the time of elution.

By comparing the number of detected peptides and the areal (MA) in the control and sample, we can conclude that the amount of *Chlamydomonas* RppH in the positive control is significantly higher than in the crude extract isolated from *C. reinhardtii* transformant four. The low content of peptides in *C. reinhardtii* can also be seen from the score value in table 4.1. When there are few peptides detected, the coverage will also be low. However comparing the samples would be meaningless since the samples are from different organisms and due to differences in purification and concentration of the sample and positive control before SDS-PAGE.

5'RppH-6xHN	Protein score	Coverage (%)	Detected RppH peptides (sequence)		
Sample	1,62	5,10	VLLAQRPVGK	GAAAADRPVR	SNAGLWEFPGGK
Control	263,77	38,78	VLLAQRPVGK	GAAAADRPVR	SNAGLWEFPGGK

Table 4.1 Mass spectrometry results for RppH-6xHN. The score and coverage values for the positive control are significantly higher than the values seen for *C. reinhardtii* transformant four, being a result of huge differences in the amount of RppH-peptides in the samples. This is however expected due to purification and concentration of the positive control sample.

4.4 The 5'*rppH*-GFP-6xHN transformation vector

In addition to analyzing the localization of *Chlamydomonas* RppH with RppH-6xHN, localization analysis using GFP (green fluorescent protein) was performed. GFP is a fluorescent protein allowing direct localization of RppH under the microscope. The construct, consisting of 5' prime sequences of *rppH* fused to the coding region of ZsGreen 1 GFP (derived from a *Zoanthus* sp. reef coral, Clontech) and terminated by histidine-tag sequences, was inserted into the same sites in the pChlamiRNA3int vector (*Chlamydomonas* Resource Center, Minnesota) as the *rppH*-6xHN construct (Figure 4.23). The chimeric *HSP70A::RBCS2* promoter::*aphVIII::RBCS* terminator sequence in the vector provides paromomycin resistance.

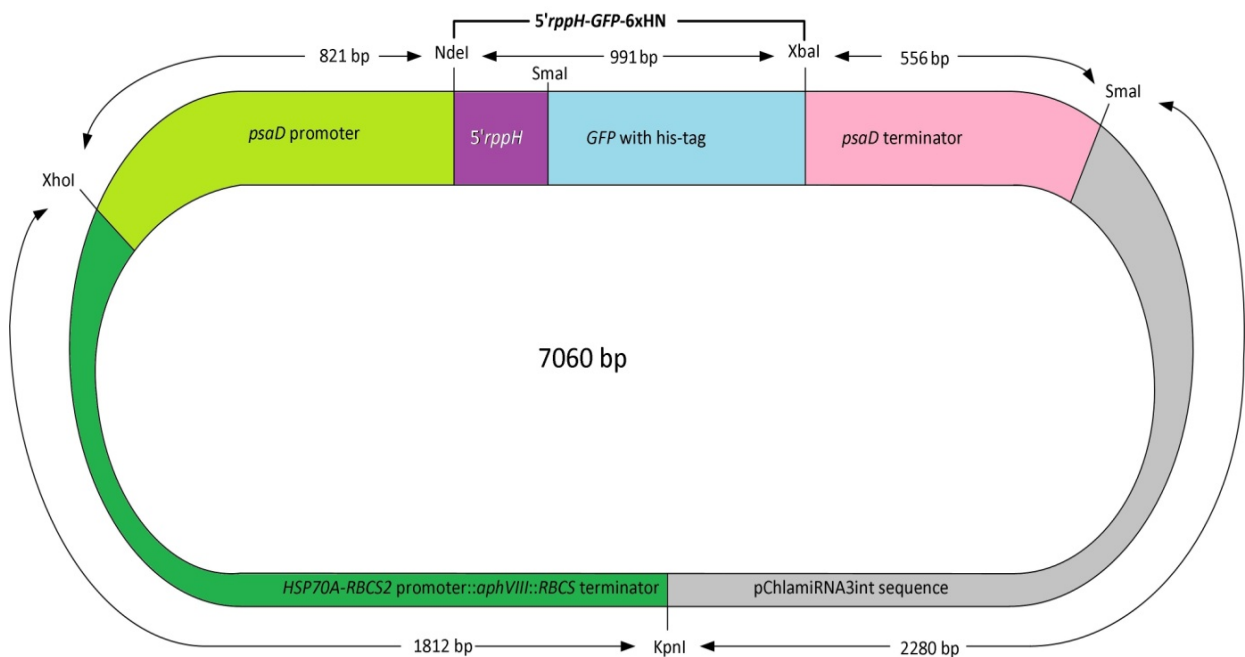


Figure 4.23 The 5'*rppH*-GFP-6xHN transformation vector. The 5'*rppH* sequence is shown in purple, while the GFP including a histidine tag is shown in blue. 5'*rppH*-GFP-6xHN is flanked by the *psaD* promoter (light green) and the *psaD* terminator (pink). The backbone sequence is shown in grey and the *HSP70A-RBCS2* promoter::*aphVIII::RBCS* terminator is shown in emerald green. Not drawn to scale.

The final transformation vector containing 5'*rppH*-GFP-6xHN was introduced into cw15 *C. reinhardtii* by nuclear transformation, and the transformants were screened using PCR. One positive transformant, hereafter referred to as GFP5, was used for further studies.

4.5 Verification of 5'RppH-GFP-6xHN transformants

4.5.1 Northern analysis

To see whether the positive GFP transformant 5 expresses the gene at the RNA level, a northern analysis was performed. RNA was isolated at the end of the dark period and in the beginning of the light, as described in materials and methods. The samples from the transformant were compared to control RNA isolated from wild-type cw15 in light and dark.

As seen in Figure 4.24, a single band is present in the RNA sample isolated from GFP5 in the dark. No band is seen in the controls, meaning that the band most likely corresponds to 5' *rppH-GFP-6xHN* transcripts. Interestingly, the 5' *rppH-GFP-6xHN* transcript is not detected in RNA isolated from GFP5 in the light, suggesting that the gene is transcribed only in small amounts or not at all.



Figure 4.24 Northern analysis of the GFP5 transformant. Two RNA samples isolated from wild-type *cw15* in light (C_L) and dark (C_D), and two RNA samples isolated from GFP5 in light (G_L) and dark (G_D) were hybridized to the random primer labeled coding region of the *GFP* gene. Only the G_D-sample contains detectable transcripts of the 5' *rppH-GFP-6xHN* gene.

4.6 5'RppH-GFP-6xHN protein localization and expression

4.6.1 Expression in *E. coli*

4.6.1.1 SDS-PAGE

To express 5'RppH-GFP-6xHN in *E. coli* the pEcoli vector (Clontech) harboring the gene was transformed into competent BL21 cells and expression induced by IPTG. Histidine-tagged 5'RppH-GFP was purified by affinity chromatography and concentrated by Amicon® Ultra Centrifugal Filters (Millipore) before loading onto an SDS polyacrylamide gel. The 5'RppH-GFP-6xHN protein has an expected size of 35 963.94 D. As seen in Figure 4.25, a band about this size is present in the lane containing 5'RppH-GFP-6xHN, a bit below the 37 kD band in the protein ladder. Due to codon optimization of the sequence for *C. reinhardtii* (done by Biruk Luelseged Abrha in the lab), 5'RppH-GFP-6xHN does not accumulate to high levels in *E. coli*.

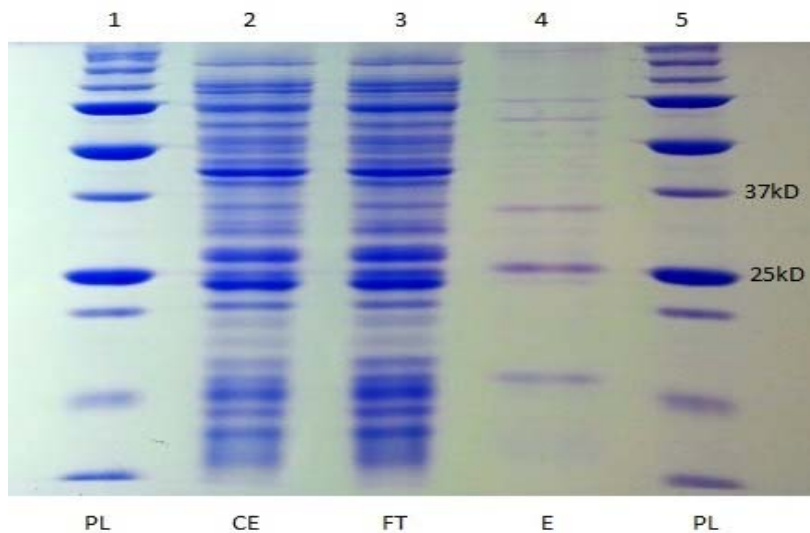


Figure 4.25 A 12% acrylamide gel showing purified 5'RppH-GFP-6xHN. The expected size of 5'RppH-GFP-6xHN is approximately 36 kD. As seen such a protein is present in the elute (lane 4) and is therefore assumed to be 5'RppH-GFP-6xHN. Crude extract (lane 2) and flow through (lane 3) are also shown. Protein ladders are seen in lanes 1 and 5.

4.6.1.2 Western blot

To verify the identity of the ≈ 36 kD band a Western analysis was done. The antibodies used are named set 3 (section 3.5.5.2), consisting of a primary ZsGreen 1 GFP antibody (Clontech) and a secondary horse radish peroxidase conjugated antibody (Sigma). As seen in Figure 4.26, the antibodies were able to detect the protein (lane 4). The signal is strong and the primary antibody seems to be fairly specific, since no signal is seen in the protein ladder. In the crude extract a weak band can be seen, equal in size to the strong signal. The band disappears in the flow through, which is expected since the flow through contains all the proteins which are not binding to the cobalt sepharose column. Concentration of 5'RppH-GFP-6xHN by the affinity chromatography explains the difference in signal strength between crude extract and elute.

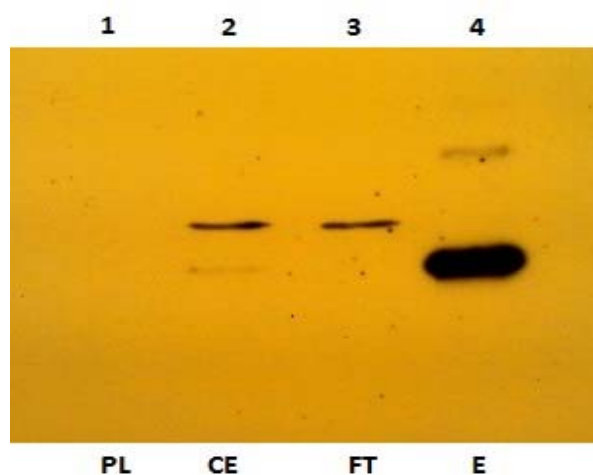


Figure 4.26 Western blot using antibodies from set 3. The antibodies detect two proteins in the crude extract (lane 2). 5'RppH-GFP-6xHN is believed to be in the lower band because its size corresponds to the size of the strong signal in the elute (lane 4). The larger band in lanes 2 and 3 is most likely unspecific, even though its signal strength is stronger than the signal from 5'RppH-GFP-6xHN. As the band is present in crude extract and flow through but not in the elute, the protein does not bind to the column. Lane 1 contains the protein ladder.

4.6.2 Expression in *C. reinhardtii*

4.6.2.1 SDS-PAGE

As mentioned we were unable to isolate chloroplasts with the protocol used. To detect the 5'RppH-GFP-6xHN protein in the *Chlamydomonas* GFP5 transformant a crude protein extract of GFP5 was fractionated on a polyacrylamide gel together with crude extract from wild-type cw15 and verified 5'RppH-GFP-6xHN isolated from *E. coli* (Figure 4.27). An extra band in the crude extract of the GFP5 transformant that could contain the 5'RppH-GFP-6xHN protein was identified (Figure 4.27) but no protein could be purified from the crude extract by Co-sepharose affinity chromatography. Western analysis and mass spectrometry were used to further analyze the proteins in the extra band.

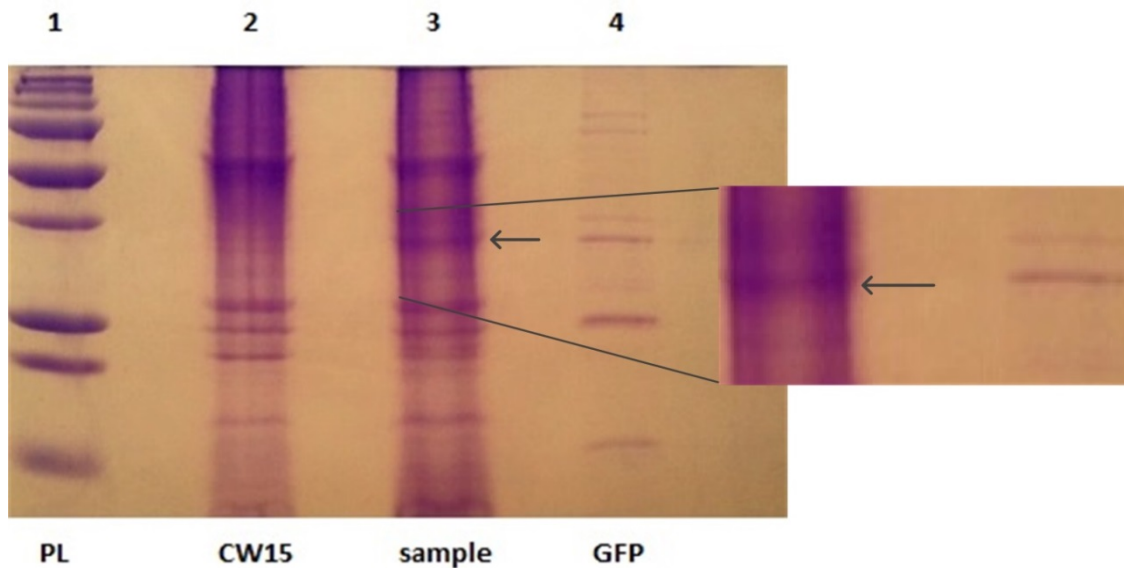


Figure 4.27 Crude protein extracts from *C. reinhardtii* fractionated on a polyacrylamide gel. A 12% acrylamide gel containing a protein ladder (lane 1), crude extract from cw15 (lane 2), crude extract from the GFP5 transformant (lane 3) and purified 5'RppH-GFP-6xHN isolated from *E. coli* (lane 4) is shown. The wild-type cw15 crude extract is a negative control while purified 5'RppH-GFP-6xHN from *E. coli* is a positive control. As mentioned previously a band around 36 kD is expected for the 5'RppH-GFP-6xHN protein. A band present in the 37 kD area in the GFP5 crude extract sample (shown with arrows and magnification) comparable in size to the band representing 5'RppH-GFP-6xHN in the positive control, could represent 5'RppH-GFP-6xHN. The band is not seen in the negative control.

4.6.2.2 Western blot

With the aim to verify the band seen in the crude extract sample from the GFP5 transformant as 5'RppH-GFP-6xHN a Western blot was performed. The antibodies used are named set 3 (section 3.5.5.2) consisting of a primary ZsGreen 1 GFP antibody (Clontech) and a secondary HRP conjugated antibody (Sigma). A LuminataTM Forte Western HRP substrate kit (Millipore) was used for detection.

Samples from an affinity chromatography were tested to see if the elute contained 5'RppH-GFP-6xHN. A Western blot is usually more sensitive than SDS-PAGE staining and should be able to detect small amounts of proteins that might not be visible on a stained polyacrylamide gel. Crude extract,

flow through and elute from the GFP5 transformant were fractionated by SDS-PAGE and a Western blot was performed (Figure 4.28). A protein was detected by the GFP antibody both in the crude extract and in the flow through but not in the elute indicating unspecific binding of the antibody to a protein in the crude extract of the GFP5 transformant.

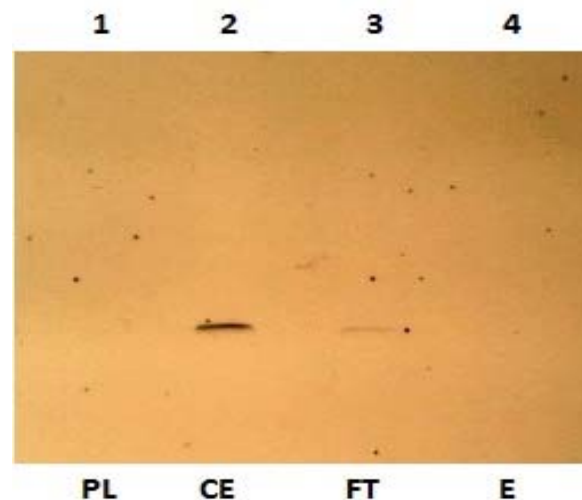


Figure 4.28. Western blot analysis of samples from the GFP5 transformant. The Western blot contains crude extract (lane 2), flow through (lane 3) and elute from affinity purification of GFP5 proteins in addition to a protein ladder (lane 1). As seen the antibodies are detecting a protein in the crude extract and in the flow through, but not in the elute. The detected protein is not binding to the sepharose column, indicating unspecific binding of the antibody.

4.6.2.3 Mass spectrometry

In a last attempt to verify the expression of 5'RppH-GFP-6xHN in the GFP5 transformant, two samples from the acrylamide gel seen in Figure 4.27 were analyzed by mass spectrometry (done by Anders Moen at the mass spectrometry group, UiO). The area containing the protein band which was believed to be 5'RppH-GFP-6xHN was cut out from the gel and digested with trypsin according to the protocol in section 3.5.6. Mass spectrometry analysis was performed both on this sample (crude extract sample from GFP5) and on a positive control (isolated 5'RppH-GFP-6xHN from *E. coli*).

From the *Chlamydomonas* sample several fractionated peptides were verified as RppH-peptides, one of them shown in Figure 4.29. (For all mass spectra obtained using the GFP5 crude extract sample see Appendix VI.) However, no GFP-peptide was detected, even though more than half of the 5'RppH-GFP-6xHN protein consists of GFP amino acid sequences. It is possible that GFP amino acid sequences are present in the sample but are difficult to ionize. Alternatively, RppH peptides may come from the endogenous RppH protein, even though it is smaller than 5'RppH-GFP-6xHN and should be located much lower in the gel. A search for peptides containing the histidine-tag was also performed, resulting in a match. Considering that both N-terminal and C-terminal 5'RppH-GFP-6xHN sequences could be detected by mass spectrometry, it appears likely that the GFP5 transformant expresses 5'RppH-GFP-6xHN. However, additional analyses are needed to confirm this conclusion.

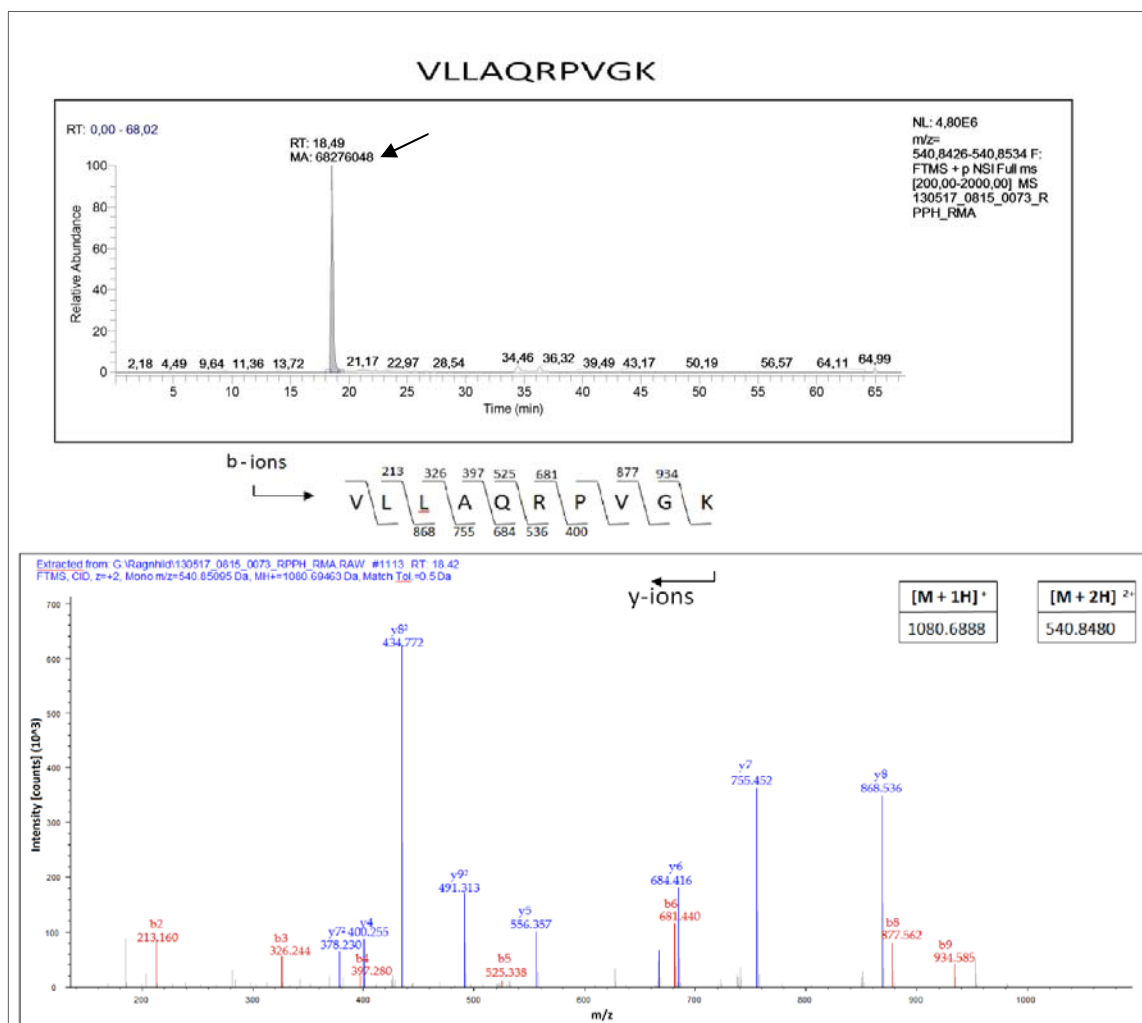


Figure 4.29 Mass spectrum of an RppH-peptide from the GFP5 transformant. Several RppH-peptides were discovered in the GFP5 crude extract sample. Since the amounts of peptides were sufficiently high, fractionated spectra of the peptides were obtained. The Figure shows one of the peptides in two spectra, the spectrum where it is detected with the proper m/z value and elution time (upper spectrum), and the spectrum where the peptide is fractionated (red peaks in the lower spectrum). For all mass spectra obtained from the crude extract GFP5 sample see Appendix VI. MA represents the area of the peaks, m/z represents mass over charge, and RT shows the time of elution.

Both the protein score and the coverage values for the control and the sample are fairly good, even though the content of RppH peptides is larger in the control compared to the sample. This is however expected since 5'RppH-GFP-6xHN was purified and concentrated from *E. coli*. Therefore the values cannot be directly compared. All the detected peptides shown are RppH-peptides. A peptide containing histidine-tag sequences was also detected (not shown).

5'RppH-GFP-6xHN	Protein score	Coverage (%)	Detected RppH peptides (sequence)		
Sample	17,68	23,98	VLLAQRPVGK	GAAAADRPVR	SNAGLWEFPGGK
Control	88,66	46,63	VLLAQRPVGK	GAAAADRPVR	SNAGLWEFPGGK

Table 4.2 Mass spectrometry results for 5'RppH-GFP-6xHN. The score and coverage values for the 5'RppH-GFP-6xHN control is higher than the values seen for the 5'RppH-GFP-6xHN sample. This is a result of differences in amount of peptides in the samples due to purification and concentration of the positive control sample. The sequences shown are from detected RppH peptides in the samples. A peptide containing the histidine-tag was also found.

4.7 RppH activity assay

4.7.1 *In vitro* RNA synthesis

To find out whether the putative *Chlamydomonas* RppH has RNA pyrophosphohydrolase activity a novel activity assay was developed. In the assay the RppH enzyme is mixed with RNA substrate, and the amount of pyrophosphate released is measured after a certain amount of time. The amount of pyrophosphate released is determined using the PPLight™ inorganic pyrophosphate assay kit (Lonza). In this kit detection of pyrophosphate is done by first converting the pyrophosphate to ATP and then determining the ATP concentration by a luciferase-based luminescence assay. The resulting relative luminescence is directly proportional to the amount of PPI present in the sample.

5' triphosphorylated RNA needed for the assay was synthesized *in vitro* from the Sk+_mod_nudix vector using the T7 promoter. Prior to RNA synthesis the template DNA was linearized with SmaI such that the size of the synthesized RNA would be about 300 nucleotides. After RNA synthesis the template was removed by treating the reaction mixture with TURBO DNase. Two RNA synthesis reactions were set up yielding approximately the same amount of RNA (Figure 4.30).

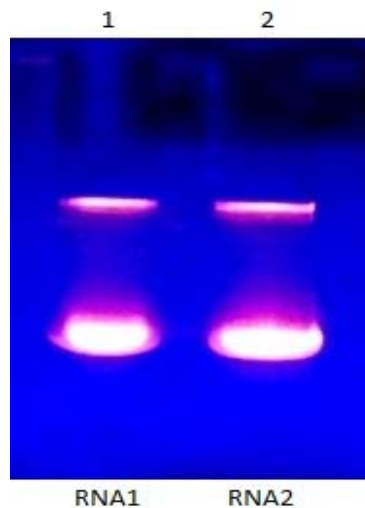


Figure 4.30 *In vitro* synthesized RNA. Samples from two synthesis reactions were fractionated on a 5% polyacrylamide gel containing urea. The expected size of the synthesized RNA is 300 nt represented by the lower bands in the gel. The upper bands might be left overs of template DNA. (There is 1µL of RNA synthesis mix in each well.)

The RNA was further purified, RNA1 with the MEGAclean™ Kit (Ambion®) and RNA2 by phenol/chloroform extraction. The final concentration of the RNA was determined in a spectrophotometer. After purification and quantification 200 ng of the samples were fractionated again on a 5% polyacrylamide gel containing urea confirming that the lower bands in Figure 4.30 were the 300 nt RNA products since the upper bands were lost in the purification.

4.7.2 RppH proteins from *C. reinhardtii* and *E. coli* used in the assay

Proteins localized to organelles normally contain N-terminal signal sequences that, if present, might interfere with the activity of the protein. We therefore thought to identify a signal peptide in the putative *Chlamydomonas* RppH protein in order to synthesize the protein without the signal peptide

for use in the activity assay. By comparing the *Chlamydomonas rppH* sequence with homologous Nudix proteins in databases, what is believed to be the transit peptide was found. Removing 98 nucleotides from the 5' end of the *Chlamydomonas rppH* gene, the gene length becomes more alike related sequences and the homology increases. A sequence of the exogenous *rppH* lacking what was believed to be the transit peptide was therefore synthesized. The sequence was cloned into a pEcoli-Nterm6xHN vector (Clontech), resulting in a 483 bp long open reading frame including an N-terminal histidine-tag. The gene is in the following referred to as synthetic *rppH* (*srppH*) and is coding for a 17 058 D large protein.

The construct was transformed into TB1 cells, followed by maxiprep, and a new transformation into BL21 protein expression cells. After induction by IPTG, sRppH was isolated from protein expression cells by affinity chromatography. The enzyme was further concentrated with Amicon®Ultra Centrifugal Filters (Millipore) and analyzed by SDS-PAGE. In addition to sRppH, RppH-6xHN was also tested for RNA pyrophosphohydrolase activity.

Two positive controls were additionally included in the activity assay. Both controls consisted of the verified RppH protein from *E. coli*, called E.coli1 and E.coli2. E.coli1 contains an N-terminal histidine-tag and was cloned in a pEcoli-Nterm6xHN vector and expressed in BL21 cells. The protein was purified by Co-sepharose affinity chromatography, concentrated, and analyzed by SDS-PAGE. The size of E.coli1 is approximately 23 kD (expected size 23331.34 D).

The second positive control, E.coli2, was purchased from New England Biolabs. E.coli2 does not contain any histidine-tag, and can be used directly in the activity assay.

The three isolated enzymes used in the assay, E.coli1, RppH-6xHN, and sRppH can be seen in Figure 4.31. All constructs contain a histidine-tag. The Figure shows the different enzymes after purification by affinity chromatography.

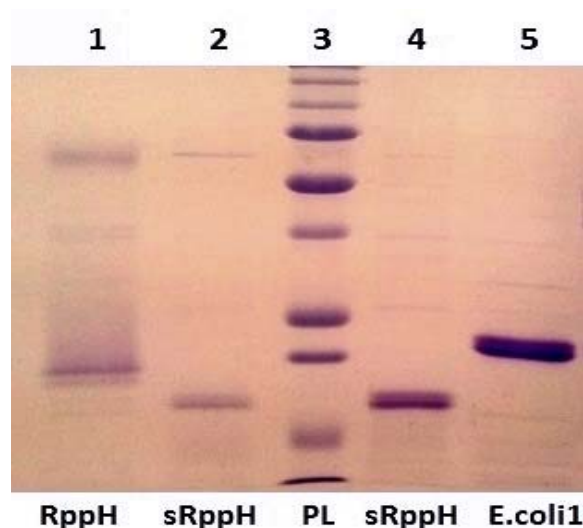


Figure 4.31 Coomassie blue-stained polyacrylamide gel showing purified histidine-tagged RppH proteins. Three of the four enzymes used in the activity assay, sRppH (lane 2 and 4), RppH-6xHN (abbreviated RppH, lane 1) and E.coli1 (lane 5) are seen. Expected size of sRppH is approximately 17 kD while a band between 20 kD and 25 kD is expected for E.coli1. The RppH-6xHN protein should have a size of approximately 20 kD. Two bands of very similar size are present in lane 5 of which the lower (stronger) band represents *E. coli* RppH.

4.7.3 RppH activities

The pyrophosphate concentration was determined by measuring luminescence in a luminometer. A standard curve was made using pyrophosphate concentrations from 0 to 10 $\mu\text{g}/\mu\text{l}$. Three different measurements were performed, and the average value for each concentration was calculated (Table 4.3).

Concentration PPI	RLU			Calculations	
$\mu\text{g}/\mu\text{L}$	Std. 1	Std. 2	Std. 3	Average RLU	Actual RLU (average)
0	82086	81491	81898	81825	0
0,078	144176	142202	144573	143650	61825
0,315	171500	168655	172534	170896	89071
0,625	390392	375756	389322	385156	303331
1,25	567191	542228	568937	559452	477627
2,5	1004876	936273	1011605	984251	902426
5	1704848	1516209	1733384	1651480	1569655
10	3056355	2585375	3137393	2926374	2844549

Table 4.3 Standard curves. The table show three different measurements performed on the same samples named Std. 1, Std. 2, and Std. 3. Luminescence is shown in relative luminescence units (RLU). The average RLU is the mean of the three measurements. The actual RLU was calculated by subtracting the average RLU of the blank (0 $\mu\text{g}/\mu\text{l}$ PPI) from the average RLU of the samples.

Based on the RLU values including the average value, four standard curves were created in Excel (Figure 4.32).

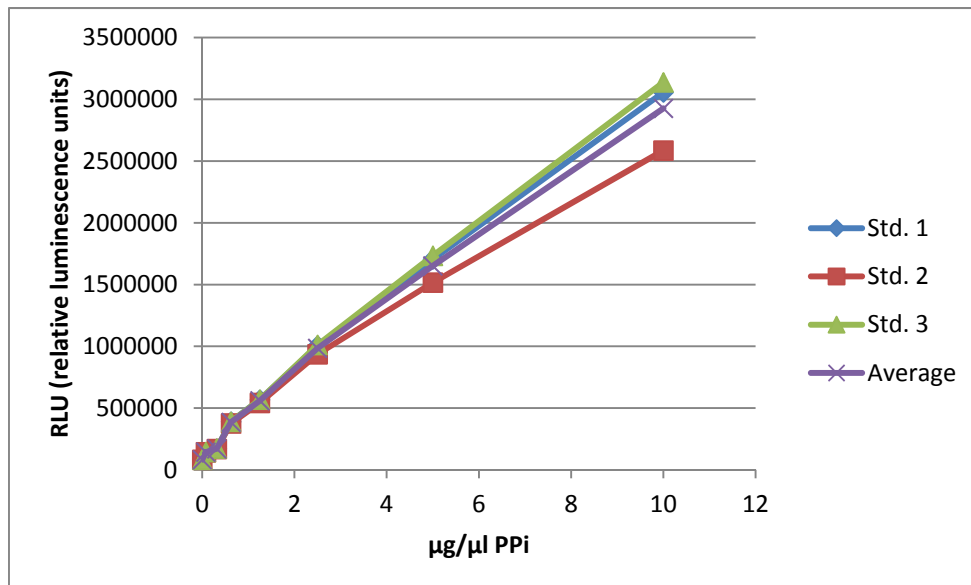


Figure 4.32 PPI standard curves. As seen all three standards (blue, red, and green) are approximately linear, indicating that the assay is working. The linearity can also be seen in the average curve (purple). Y-axis, RLU from Table 4.3; x-axis, PPI concentrations.

The standard activity assay for *E. coli* RppH from NEB and the original RppH assay described by Deana et al. (2008) both use radiolabeled RNA and are rather complicated. Using the PPI assay the relative RNA pyrophosphohydrolase activity of E.coli2, E.coli1, sRppH, and RppH-6xHN could be determined relatively easily (Table 4.4; Figure 4.33). The activity of E.coli2, i.e. RppH purchased from NEB, can be used as reference. All samples contained 2µg of *in vitro*-synthesized RNA purified with the MEGAclean™ kit (Ambion®) (RNA1). From the measured values the real relative activity was calculated by subtracting the relative luminescence units (RLU) measured in the control from the RLU measured in the different samples.

	Sample	Enzyme	Comment	RLU	Activity
1	Control	No		35059	0
2	E.coli2 (NEB)	1 µl	Undiluted	57829	22770
3	E.coli1	40-fold	Diluted	86242	51183
4	E.coli1	200-fold	Diluted	63506	28447
5	E.coli1	400-fold	Diluted	57041	21982
6	E.coli1	40-fold	Denatured	72747	37688
7	sRppH	10-fold	NEB buffer 2	38130	3071
8	sRppH	10-fold	Dilution buffer	36916	1857
9	sRppH	10-fold	Denatured	38649	3590
10	RppH	10-fold	NEB buffer 2	43821	8762
11	RppH	10-fold	Dilution buffer	49887	14828
12	RppH	10-fold	Denatured	45826	10767

Table 4.4 RNA pyrophosphohydrolase activity measurements for different RppH proteins. All samples were incubated for 30 minutes at 37°C in 1X NEB buffer 2 and released PPI was determined with the PPI detection kit (Lonza). Different dilutions of E.coli1 (in dilution buffer) were tested. No activity could be measured with undiluted E.coli1 RppH most likely due to inhibiting factors present in the sample. All proteins were diluted in enzyme dilution buffer except samples 7 and 10 which were diluted in 1X NEB buffer 2. Samples 6, 9, and 12 were denatured (5 min in boiling water) prior to PPI determination. The control contained the same components as the samples but no RppH protein.

*E. coli*1 has the highest activity of all the tested enzymes, and most activity is detected when *E. coli*1 is diluted 40-fold (corresponding to approximately 75 ng of protein in the assay). The protein's activity exceeds *E. coli*2 purchased from New England Biolabs of which approximately 50 ng were used in the assay. The only difference between the proteins is an N-terminal histidine-tag not being present in *E. coli*2. A reason for the difference in RNA pyrophosphohydrolase activity is difficult to explain, however while *E. coli*2 was purchased (NEB) *E. coli*1 was freshly purified in our lab. Both enzymes work as positive controls in the assay. sRppH did not show any RNA pyrophosphohydrolase activity, while the activity of RppH-6xHN, abbreviated RppH is about half of the NEB enzyme. RppH-6xHN contains a C-terminal histidine-tag while sRppH has the histidine-tag at its N-terminus. RppH-6xHN does also contain what is believed to be the transit peptide, while the sequence is removed from sRppH. To fully verify RppH as an RNA pyrophosphohydrolase additional analysis need to be performed with RppH-6xHN. Alternatively it is possible to remove the histidine-tag from sRppH by using enterokinase, and test if this renders the protein active.

Activity assay

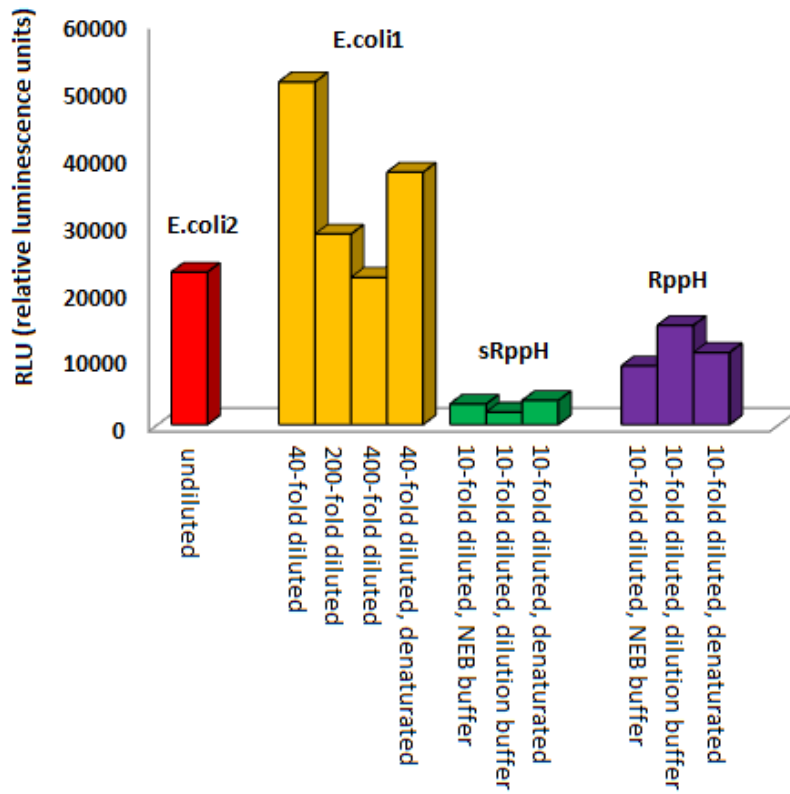


Figure 4.33 RNA pyrophosphohydrolase activities of RppH proteins. Block diagram of the activity values for E.coli1 (red), E.coli2 (yellow), sRppH (green) and RppH-6xHN abbreviated RppH (purple) seen in Table 4.4. E.coli1 is the most active protein, while E.coli2 and RppH have some RNA pyrophosphohydrolase activity. sRppH is inactive.

5 DISCUSSION

With the aim to localize the *C. reinhardtii* RppH homologue, an *rppH*-6xHN construct was stably inserted into the nuclear genome of the cell wall-less mutant cw15. A positive transformant was isolated and verified at the DNA level. The cell line was purified by repeated growth on paromomycin-containing agar plates and by growth in paromomycin-containing liquid medium. Further the transformant has been shown by RT-PCR to most likely produce the corresponding transcript. mRNA abundance is however quite low and difficult to show in an RNA blot. The same was seen for GFP5, a verified 5' *RppH*-GFP-6xHN transformant containing the same regulatory sequences for transgene transcription. Both transformants were analyzed for protein expression by SDS-PAGE and mass spectrometry. For the GFP5 transformant accumulation of 5' *RppH*-GFP-6xHN was also analysed by Western blotting. Finally a novel activity assay was developed in order to verify the RppH homologue from *C. reinhardtii* as an RNA pyrophosphohydrolase.

5.1 The translation start site of *Chlamydomonas rppH*

As previously mentioned, the translation start site of our construct differs from the site in the annotation of the gene on the phytozome website. It is difficult to know if the correct translation start site was chosen. We can, however, investigate whether the Nudix motif in the protein is intact by using our chosen RppH protein as query sequence in a BLAST search. As seen in Figure 5.1 none of the different domains are starting until amino acid number 50. In other words no sequence crucial for the protein function is missing.

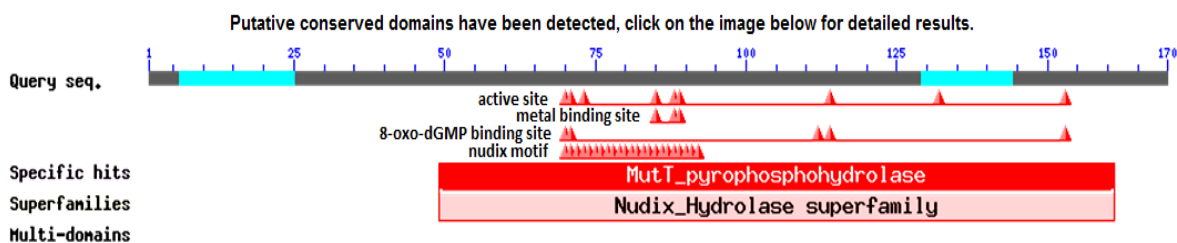


Figure 5.1 Putative conserved domains in *Chlamydomonas* RppH. Screen shot from an RppH BLAST search showing the active sites, the metal binding sites, 8-oxo-dGMP binding sites, and Nudix motif in the amino acid sequence.

It can also be noted that the N-terminal sequence not being crucial for protein function, is consistent with our hypothesis of the chloroplast as location for RppH, since the protein could harbor an N-terminal signal peptide. Another result of the BLAST search that is supporting the notion of *Chlamydomonas* RppH being a chloroplast protein is the list of proteins showing most homology to *Chlamydomonas* RppH. All are bacterial proteins with homology starting after amino acid number 30. No RNA-binding site is detected by BLAST but BLAST also fails to detect an RNA-binding site in the sequence of *E. coli* RppH.

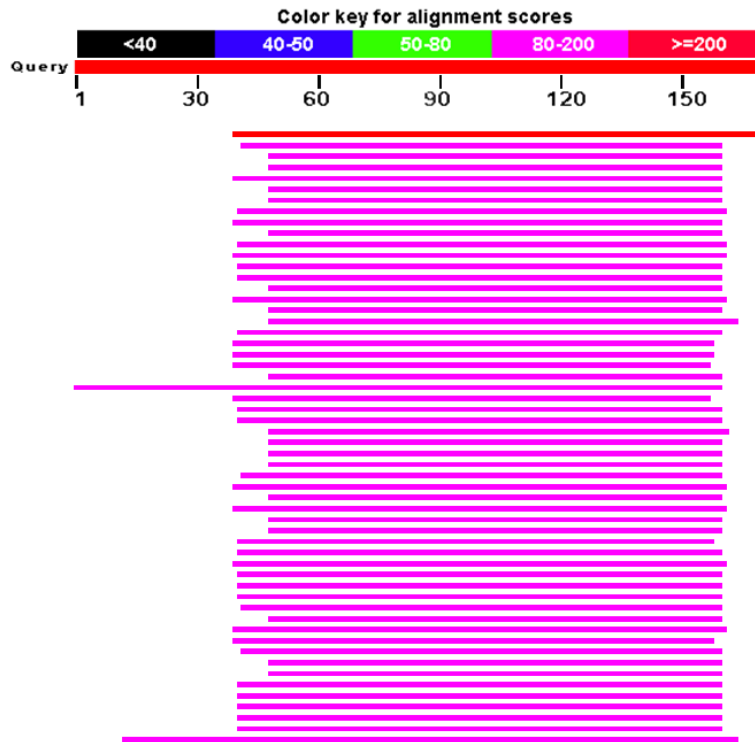


Figure 5.2 RppH BLAST search. Screen shot from a blast performed with the RppH protein sequence, showing aligned homologous protein sequences, mostly from bacteria. The six most homologous proteins are shown in Table 5.1.

Protein	Organism	E value
Hypothetical protein CHLREDRAFT 107572	<i>Chlamydomonas reinhardtii</i>	2,00E-87
Mutator MutT protein	<i>Paracoccus</i> sp. TRP	1,00E-48
Mutator MutT protein	<i>Roseovarius</i> sp. 217	4,00E-47
Mutator MutT protein	<i>Paracoccus dentrificans</i> PD1222	9,00E-47
NTP pyrophosphohydrolase	<i>Agrobacterium albertimagni</i>	1,00E-46
Mutator MutT protein	<i>Roseovarius</i> sp. TM1035	2,00E-46

Table 5.1 Proteins homologous to *Chlamydomonas* RppH. The six proteins showing most homology to *Chlamydomonas* RppH in a BLAST search are shown. All are bacterial proteins, except the hypothetical CHLREDRAFT 107572 protein, being the endogenous *C. reinhardtii* RppH sequence.

5.2 Verification of transformants at the DNA level

The transformants were screened and verified at the DNA level by PCR. For the RppH-6xHN transformant four cell line Southern analysis was also performed, but without success. It is not clear why transformant four was positive in four different PCRs but not in Southern analysis. It is possible that the constructs were integrated into the genome such that the enzymes chosen for the Southern analysis did not cut as expected or that the radio-labeled probe was not good. Our main suspicion was however that the cells were contaminated with untransformed cells at the time of Southern analysis. The cells therefore had to be purified by additional growth on paromomycin plates and in paromomycin containing liquid medium.

5.3 Growth of *C. reinhardtii* in paromomycin-containing liquid media

Growth of positive transformants in liquid HS media containing different concentrations of paromomycin was performed in an attempt to purify transformed cells due to contradictory PCR and Southern analysis results. We suspected that untransformed *C. reinhardtii* cells might be capable of growing on paromomycin plates nearby paromomycin resistant transformants. Since the colonies are picked and grown in paromomycin-free liquid media, non-resistant cells might outgrow real transformants if the colony is a mixture of transformed and untransformed cells. In such a case verification of a transformant at the DNA level would lead to inconclusive results.

C. reinhardtii tolerates only small amounts of paromomycin in liquid medium. This could be due to insufficient expression of *aphVIII*. The paromomycin resistance gene is under control of a heat shock promoter and the cells were therefore initially incubated at 37°C. The *aphVIII* promoter *HSP70A-RBCS2* is an especially strong promoter after heat shock (Wu et al. 2008). The most likely reason for cell death of transformed *C. reinhardtii* cells in paromomycin-containing liquid HS medium is increased diffusion of paromomycin. Paromomycin inactivates translation in the chloroplast and resistant cells have to modify paromomycin in the cytosol before the molecule enters the chloroplast. At high concentrations of paromomycin some molecules might escape modification and block chloroplast translation.

5.4 Verification of transformants at the RNA level

Several northern blots were performed in order to detect 5'psaD-*rppH*-6xHN-psaD3' and 5'psaD-*rppH*-GFP-6xHN-psaD3' transcripts. For both constructs detection turned out to be difficult.

The results obtained in Northern analysis show that transcript levels of especially the 5'psaD-*rppH*-6xHN-psaD3' constructs (Figure 4.14) are low if present at all, contradicting the results seen in SDS-PAGE, where a band believed to be the expressed *rppH*-6xHN transgene is visible. A possible explanation might be that the RNA isolations were performed at time points in the light-dark cycle where transcription is low. Another explanation would be that the transgene is poorly transcribed in the transformants, and that the protein band seen in SDS-PAGE represents a wild-type *C. reinhardtii* protein.

Transcripts could be detected by reverse transcription on total RNA isolated from RppH-6xHN transformant four (Figure 4.15) which might be due to lower detection limits using this method. However, since no sequencing result is available for the amplified fragment, the PCR product may be unspecific.

5'psaD-*rppH*-GFP-6xHN-psaD3' transcripts could be detected by Northern analysis in RNA isolated from transformant GFP5 at the end of the dark cycle (Figure 4.24), corresponding more to the

strength of the protein band believed to be 5'RppH-GFP-6xHN in results achieved from SDS-PAGE (Figure 4.27).

5.5 Transgene transcription in *C. reinhardtii*

Both the *rppH*-6xHN and the 5'*rppH*-GFP-6xHN transgenes are under control of the *psaD* promoter and terminator and should therefore in principle be transcribed in the same way as the *psaD* gene. However transcription of transgenes may be differently regulated and can be affected by the insertion site of the construct in the genome, mRNA stability, inefficient RNA processing and gene silencing. For a long time a major obstacle to research in *C. reinhardtii* has been the poor expression of transgenes from the alga's nuclear genome. Good transformation protocols exist and transformants are also readily obtained but it has been difficult to identify clones that express the gene of interest at reasonably high levels (Fuhrmann et al. 1999, Schroda et al. 2000). Low transgene expression in *C. reinhardtii* is not understood on a molecular level but possible mechanisms may be non-conventional epigenetic suppression activities and/or compact chromatin structures. In some cases the problem has been alleviated by using specialized promoters, as *psaD*, and codon optimization but no general solution has been found. Another possibility for solving the problem might be to use a genetic screening system developed by Neupert et al. (2009) that allows selection of high-level transgene expression mutants.

Expression of *C. reinhardtii* nuclear genes may also be regulated by intron sequences. A 30-fold increased expression from the *rbcS* promoter has for instance been observed by including the first *rbcS* intron into a *Ble* coding sequence (Lumbreras et al. 1998). The *psaD* gene however does not harbor any introns and regulatory sequences must therefore lie in the promoter and/or the untranslated regions (Fischer and Rochaix 2001). Fischer and Rochaix (2001) reported efficient expression of both endogenous and exogenous selectable markers using a *psaD*-based vector. Based on these results the insertion of the intron in the constructs should not be necessary. The transformation vector used in this thesis originally contained the *rbcS* intron but the sequence was lost in the final cloning step.

5.6 Chloroplast isolation

To localize RppH in *C. reinhardtii*, we wanted to isolate chloroplasts in order to compare the relative amounts of RppH in cells and chloroplasts. However, the procedure used failed in breaking of the cells by pushing them through a needle (Figure 4.20). To be able to break the cells with a needle and syringe in such a way as described in the protocol, the cells need to be of approximately the same size. This should in theory be achieved by growing the cells in 12-hours light/12-hours dark cycles. When the transformants were checked under a microscope before starting the protocol, it was however seen that the sizes of the cells were unequal probably due to differences in growth conditions compared to the protocol.

Another important aspect of the protocol is to use cell wall-less mutants. Cw15 is described as a mutant containing cell-wall structures of the inner and outer wall. It is still mentioned in the protocol

as a strain possible to use, though. To test whether the mutant is cell wall-less we tried to lyse the cells with digitonin. Digitonin will destroy the cell membrane but is incapable of breaking cells containing a cell wall. The cells did not lyse showing that the cw15 strain is not completely cell wall-less.

An additional problem was the Percoll gradient, and the fact that intact cells did not move through 65% Percoll upon centrifugation as anticipated (see section 3.4.7). We therefore decreased the percentage of Percoll to 50%. The cells were able to go through indicating that our cells were less dense compared to the cells used in the protocol. Again differences in growth conditions are the most likely explanation for varying behavior of the cells in the isolation procedure.

5.7 Verification of 5'*rppH-GFP-6xHN* and *rppH-6xHN* transgene expression in *C. reinhardtii*

As previously mentioned transcription of *psaD* is initiated by light and we therefore assume that expression of the PsaD protein is highest during the light period of the day. Because of these assumptions we chose to isolate proteins from transformants approximately four hours into the light period (having them on 12-hours light/12-hours dark cycle). In SDS-PAGE analysis of the GFP5 transformant a protein band containing the approximate size of 5'RppH-GFP-6xHN was observed (Figure 4.27). This putative 5'RppH-GFP-6xHN protein seemed to be highly expressed. The band could however not be confirmed as 5'RppH-GFP-6xHN due to inconclusive results obtained from Western blots and mass spectrometry (Figures 4.28 and 4.29).

A band that could be RppH-6xHN protein was also seen in SDS polyacrylamide gel electrophoresis using a crude extract sample from *C. reinhardtii* transformant four (Figure 4.21). However, mass spectrometry excluded the protein band being RppH-6xHN because the amount of RppH-6xHN in the sample simply did not match the strength of band seen in the stained gel. Small amounts of RppH-peptides were detected by mass spectrometry in the transformant (Figure 4.22), but it was not possible to decide whether they originated from endogenous RppH or RppH-6xHN. The proteins were most likely located close to each other in the polyacrylamide gel.

A difference in transgene expression may occur if one of the proteins is toxic for the cell. For instance the RppH-6xHN protein might be active and function as an RNA pyrophosphohydrolase despite the histidine tag. In the pyrophosphate activity assay more activity was for instance measured for RppH-6xHN compared to sRppH, where the presumed transit peptide had been removed (Figure 4.33). The *psaD* promoter is also a constitutive promoter, which in the case of RppH-6xHN being a toxic compound, would be a disadvantage compared to using an inducible promoter.

Another reason for differences in transgene expression could be that the *rppH-6xHN* transcript is unstable. Different transcript levels were seen by northern analysis in the two transformants, where 5'*psaD-rppH-6xHN-psaD3'* transcripts in contrast to 5'*psaD-rppH-GFP-6xHN-psaD3'* transcripts were not detectable at all (Figures 4.14 and 4.24). To fully verify both transformants at the protein level additional analyses must be performed.

5.7.1 Western analysis

5.7.1.1 Specificity and detection of RppH-6xHN using histidine-tag antibodies

Originally we wanted to localize the RppH-6xHN by immunohistochemistry using the histidine-tag. However no signal was observed in Western analysis using RppH-6xHN purified from *E. coli*. Even though two different histidine-tag antibodies were used, detection was not achieved (Figures 4.18 and 4.19). A possible explanation, which was however ruled out by mass spectrometry, could have been that the protein purified from *E. coli* was not RppH-6xHN or that its histidine-tag had been lost. The more likely explanation is that the antibodies did not work properly. This was in some way supported by unspecific binding of the antibodies. Studies reporting variability in the immunodetection of histidine-tagged recombinant proteins support this conclusion (Debeljak et al. 2006). Debeljak et al. (2006) showed that detection levels of his-tagged proteins largely depended on the particular histidine-tag antibody used. The article further reported different detection levels of the positive control, in addition to unspecific detection, which is consistent with our result. Four histidine-tag antibodies were investigated, only one was able to specifically detect the histidine-tagged proteins tested.

Other options are that the histidine-tag is unaccessible to the antibodies, but this is unlikely due to denaturation of the protein prior to SDS-PAGE.

5.7.1.2 Detection of 5'RppH-GFP-6xHN using a GFP primary antibody

Expression of 5'RppH-GFP-6xHN in the GFP5 transformant could be neither confirmed nor disproved in the Western blot results obtained. Two different batches of GFP primary antibodies were used, showing differences in protein detection in the *C. reinhardtii* samples. However due to time limits we were unable to perform additional Western blots, which most likely would have clarified if 5'RppH-GFP-6xHN is expressed or not. The primary GFP antibody seemed to be sensitive to repeated freezing and thawing cycles which may have rendered the antibody inactive over time. To fully confirm expression of 5'RppH-GFP-6xHN positive and negative controls should be included in the analysis. Alternatively additional analysis using mass spectrometry may also be performed.

5.7.2 Verification of 5'RppH-GFP-6xHN and RppH-6xHN by mass spectrometry

Generally mass spectrometry analyses are conclusive, however it might be difficult to specifically confirm transgene expression if the transformant harbors a similar protein. The only difference between RppH-6xHN and endogenous RppH is the C-terminal histidine-tag, and without detection of a peptide containing histidine-tag sequence, the expression of RppH-6xHN in the *C. reinhardtii* transformant can not be confirmed. As mentioned a few peptides containing RppH-sequences were detected in the transformant but the histidine-tag was not observed (Table 4.1). However, supporting transgene RppH-6xHN expression is that the protein was cut out of the acrylamide gel in

the 20kD area. The size of the exogenous RppH protein should be a bit smaller. This is however not proof for transgene expression since cutting the gel is not very precise.

Confirmation of 5'RppH-GFP-6xHN expression in the GFP5 transformant was easier, since more than half of the protein consists of GFP. However, no peptides containing GFP sequences were obtained (Table 4.2). A possible explanation might be that the sequence is difficult to ionize and therefore not detectable in mass spectrometry. A second possibility is that the peptides detected originate from endogenous RppH. A search detecting peptide sequences containing the histidine-tag confirmed however that the detected protein is 5'RppH-GFP-6xHN. Furthermore the endogenous RppH protein is much smaller than 5'RppH-GFP-6xHN. Again, additional analysis should be performed to confirm the results, especially since the Western blots were inconclusive.

5.8 Determination of the RNA pyrophosphohydrolase activity of *Chlamydomonas* RppH using a novel activity assay

The protein activity assay has so far not given a clear answer to whether the protein truly is an RNA pyrophosphohydrolase. Increased levels of pyrophosphate have been measured several times, but results are not reproducible. It should also be noticed that the two enzymes containing an N-terminal histidine-tag in the assay, E.coli1 and sRppH, show huge differences in RNA pyrophosphohydrolase activity (Figure 4.33). E.coli1 is the enzyme in the assay that shows most activity, while sRppH is not active.

Buffers may be tested in addition to other factors for improving the activity assay. The assay is also dependent on pure solutions, which might affect the results. For instance if RNases are present the RNA template will be degraded, giving false positive results. The opposite may happen if a pyrophosphatase or ATPase is present, degrading the substrates used by the luciferase to produce light. To be sure that the assay is working properly, the results should be confirmed by multiple measurements.

As mentioned, RppH is a hypothetical protein, and may not possess the activity and function in *C. reinhardtii* which is anticipated in this thesis. First we have not yet proved that the protein is located in the chloroplast, an important assumption if the protein truly is a homolog of the RppH protein found in *E. coli*. Second assuming that the protein is located in the chloroplast it can still harbor other enzymatic activities. Third it is not shown that the sequence actually codes for a functional protein. The sequence may in other words possess several other functions or none at all. However, we know for certain that the sequence codes for a protein containing a MutT Nudix motif.

6 CONCLUSION

An RppH-6xHN construct has been cloned and analyzed in *C. reinhardtii*. Additional analyses of a previously cloned GFP5 transformant have also been performed. Both clones might express their transgenic protein but further verification is necessary. Determination of RppH localization in *C. reinhardtii* using the RppH-6xHN construct was not possible, due to failure in chloroplast isolation and in antibody detection of the histidine tag. An activity assay has also been developed to test the activity of RppH. The assay worked well with *E. coli* RppH but did not give reproducible results with the putative *Chlamydomonas* RppH.

7 FURTHER WORK

The work in this thesis has led to interesting results which might lead to localization of a putative RppH protein in *C. reinhardtii*. However several aspects should be considered to improve the results.

First of all the tested transformants should be further analysed to clarify their transgene expression. Mass spectrometry might be performed using more sample volume to be able to detect peptides containing the entire sequences of the transgenes. It is also necessary to do new Western blots to verify the presence of 5'RppH-GFP-6xHN in GFP5.

New transformants should be screened to find cell lines with high transgene expression, e.g. using the screening system developed by Neupert et al. (2009). Even though the *psaD* promoter used in this project has been shown to be a strong promoter (Fischer and Rochaix 2001) other promoters may be tested. To include the *rbcS2* intron in the construct might also be considered, as the intron often is used as a transcriptional enhancer (Lumbreras et al. 1998).

To improve the Northern blot results RNA could be isolated at different time points, e.g. continuously every second hour for 24 hours, to determine at which time point transgene transcription is highest. Alternatively qRT-PCR may be considered.

To localize RppH in *C. reinhardtii* confocal microscopy should be performed using a verified 5'RppH-GFP-6xHN transformant. The RppH-6xHN construct may also be used to localize RppH in isolated chloroplasts if a working protocol for chloroplast isolation is found. The problem with the tested protocol was breaking the cells, most likely because of residual cell-wall elements of the cw15 mutant. This problem could be avoided by producing protoplasts with autolysine. Autolysine can be isolated by fusing synchronized compatible *C. reinhardtii* gametes (Schlösser et al. 1976). In addition, the Percoll gradient and the density of the cells should be investigated to improve the separation of chloroplasts from intact cells.

Last the RppH activity assay can be optimized to increase its sensitivity in order to detect even low amounts of RppH activity.

Appendix I: Abbreviations

AMP	Adenosine-5'-monophosphate
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
<i>atpB</i>	Coding for ATP synthase complex, subunit β
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
cm	Centimeter
CSP41	Endoribonuclease, possible transcription factor
CTP	Cytidine triphosphate
D	Dalton
DEPC	Diethylpyrocarbonate
dH ₂ O	Distilled H ₂ O
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g	Gravitational force
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
Hsp70	Heat shock protein 70
IPTG	isopropylthio- β -galactoside
kb	Kilobase pair
kD	Kilodalton
L	Liter
M	Molar concentration
mA	Milliampère
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar concentration
MOPS	3-morpholinopropane-1-sulfonic acid
mRNA	Messenger RNA
N	Normality
ng	Nanogram
nt	Nucleotide
nm	Nanometer
Ntr-PAP	Nucleotidyl transferase family; poly(A) polymerase
O.D	Optical density
PCR	Polymerease chain reaction
<i>petA</i>	Coding for cytochrome f
PNPase	Polyribonucleotide polymerase; 3'-5' exoribonuclease
pmol	Picomol
<i>psaD</i>	Coding for Photosystem I subunit D

<i>RBCS2</i>	Coding for Ribulose Bisphosphate Carboxylase/Oxygenase Small Subunit 2
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RNase	Ribonuclease
s	Second
SRP	Signal recognition particle
Tat-system	Twin-arginine translocatin system
TEMED	Tetramethylethylenediamine
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
u	Units
UTP	Uridine-5'-monophosphate
UTR	Untranslated region
UV-light	Ultraviolet light
V	Volt
w/v	Weight by volume
w/w	Weight by weight
μCi	Microcurie
μg	Microgram
μL	Microliter

Appendix II: Transformation vector sequence

pChlamiRNA3 vector with Nudix-Cterm-His sequence

5'UTR-red

rppH-purple

3'UTR-green

Histidine-tag- orange

HSP70A-RBCS2 promoter::*aphVIII*::*RBCS2* terminator sequence -blue

Bold black letters- enterokinase cleavage site

Underlined bold letters- primer binding sequences

Underlined letters with yellow background- restriction sites used in cloning

UPPER CASE LETTERS- INSERTED SEQUENCE (*rppH*, histidine-tag, additional sequence)

Lower case letters- vector backbone

```
1      TATGGACGAG GGAGACGCGC CCGCTGCCGC CCCTGGTGCC GTTCGGCTGC TTGTGGTGGT
61     GGGTGTGGTC CTGCTGGACG ACCCACTGTG GGACCACGAG ACGGGCGAGC CCAGCAAGGG
121    CGCCGCCGCGC GCGGATCGGC CTGTGCGGGT GCTGCTGGCT CAGCGGCCTG TGGGCAAGAG
181    CAACGCGGGG CTGTGGGAGT TCCCGGGCGG CAAGGTGGAC CCAGGGGAGA CGCCGAGGC
241    GCGCTGGTT  CGCGAGCTGT ATGAGGAGCT GGGCATCTCG GTGGACCCGG CGGACCTGGC
301    GCCGCTCACA TTTGCCTCCC ACACCTACCC CACCTTCCAC CTGCTCATGC CGCTGTATGC
361    CTGCCGGCGC TGGTCGGGCG TGCCTGTGGG CGCGGAGGGC CAGGCGGTGG CGTGGGCGGC
421    TGCGGGCGAG GTGACGTCTT TCAACCTGAC GCCTGCAGAC ATACCGCTGG TACCGCTGT
481    GCTGGCGGCT ATGCGGCACT ACCCAGCA GGTCCGCCGC CTGGTTCCGC GTGGCTCTCC
541    GGGCGCTGCA GGTCATAATC ATAATCATAA TCATAATCAT AATCACAAT AATTAATTAA
601    TCTAGAtggc agcagctgga cgccctgtac catggagaag agctttactt gccgggatgg
661    ccgatttcgc tgattgatac gggatcggag ctcggaggct ttcgcgctag gggctaggcg
721    aagggcagtg gtgaccaggg tcgggtgtggg gtcggccac ggtcaattag ccacaggagg
781    atcaggggga ggtaggcacg tcgacttggt ttgcgacccc gcagttttgg cggacgtgct
841    gttgtagatg ttagcgtgtg cgtgagccag tggccaacgt gccacaccca ttgagaagac
901    caaccaactt actggcaata tctgccaatg ccatactgca tgtaatggcc aggccatgtg
961    agagtttgc gtgcctgcgc gcgcgccggg gcgcgagttt agctgaccag cgtgggatg
1021  atgcacgcat ttgcaaggac agggtaatac cagcagcaac atggtgggct taggacagct
1081  gtgggtcagt ggacggacgg caggggaggg acggcgcagc tcgggagaca gggggagaca
1141  gcgtgactgt gcaatgcggc cgccaccgcg gtggagctcc aattcgccct atagtgagtc
1201  gtattacgcy cgctcactgg ccgtcgtttt acaacgtcgt gactgggaaa accctggcgt
1261  taccacaact aatcgcttg cagcacatcc cccttcgcc agctggcgta atagcgaaga
1321  ggcccgcacc gatcgccctt cccaacagtt gcgcagcctg aatggcgaat gggacgcgcc
1381  ctgtagcggc gcattaagcg cggcggtgtg ggtggttacg cgcagcgtga ccgctacact
1441  tgccagcgcc ctagcgcgcc ctcctttcgc ttcttcctct tcctttctcg ccacgttcgc
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1561  acggcacctc gacccccaaa aacttgatta gggtgatggt tcacgtagtg ggccatcgcc
1621  ctgatagacy gthtttcgce ctttgacggt ggagtccacg ttctttaata gtggactctt
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1801  ttttaacaaa atattaacgc ttacaattta ggtggcactt ttcggggaaa tgtgcgcgga
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1921  ccctgataaa tgcttcaata atattgaaaa aggaagagta tgagtattca acatttccgt
1981  gtcgccttha ttccccttht tgccgcattt tgccctcctg tttttgctca cccagaaacg
2041  ctggtgaaag taaaagatgc tgaagatcag ttgggtgcac gagtgggtta catcgaaactg
2101  gatctcaaca gcggttaagat ccttgagagt tttcgccccg aagaacgtht tccaatgatg
2161  agcactthta aagthctgct atgtggcgcg gtattatccc gtattgacgc cgggcaagag
2221  caactcggtc gccgcataca ctattctcag aatgacttgg ttgagtactc accagtcaca
2281  gaaaagcatc ttacggatgg catgacagta agagaattat gcagtgtgc cataaccatg
2341  agtgataaca ctcgggcaa cttactctg acaacgatcg gaggaccgaa ggagctaacc
2401  gctthtttgc acaacatggg ggatcatgta actcgccttg atcgttggga accggagctg
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2521  ttgcaaaac tattaactgg cgaactactt actctagctt cccggcaaca attaatagac
2581  tggatggagg cggataaagt tgcaaggaca cttctgcgct cggcccttcc ggctggctgg
2641  tttattgctg ataaatctgg agccggtgag cgtgggtctc gcggtatcat tcgagcactg
2701  gggccagatg gtaagccctc ccgtatcgta gthtatctaca cgacggggag tcaggcaact
2761  atggatgaac gaaatagaca gatcgctgag ataggtgcct cactgattaa gcattggtaa
2821  ctgtcagacc aagthttactc atatatactt tagattgatt taaaacttca tttttaattt
2881  aaaaggatct aggtgaagat cctthttgat aatctcatga ccaaaatccc ttaacgtgag
```

2941 ttttcggttc actgagcgtc agaccccgta gaaaagatca aaggatcttc ttgagatcct
3001 ttttttctgc gcgtaatctg ctgcttgcaa acaaaaaaac caccgctacc agcgggtggtt
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3361 ctgagataacc tacagcgtga gctatgagaa agcggccacgc ttcccgaagg gagaaaggcg
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3961 cacacaggaa acagctatga ccatgattac gccaaagcgc caattaacc tcaactaaagg
4021 gaacaaaagc tgggtac cg cttcaatac gcccagccc cccatggaga aagaggccaa
4081 aatcaacgga ggatcgttac aaccaacaaa attgcaaac tcttccgctt tttacgtgtt
4141 gaaaaagact gatcagcag aaacggggag ctaagctacc gcttcagcag ttgagagcag
4201 tatcttccat ccaccgcctt tcgtcagggg gcaaggctca gatcaacgag gcctccatt
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4321 ccagccacca acaccatcag gtccctcaga agaactcgtc caacagccgg taaaacgcca
4381 gcttttcctc cgataccgcc ccatcccacc cgcgcccgt ctcccgcagg aacgccgcgg
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4501 gatcggagtg ccggtccgca cgcccgacc gccccacgt gatcagccc gtcacctcgc
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4681 tccgctcctc gtccagatcc tccaagtoga cgctcccttc agcgacagca cgggcccctt
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5281 gcaagtcata tctgcaagca cgctgcctga tccgcggggc ttgctcgtcg actcacctgg
5341 ccatttttaag atggtgagtg acttctcttg taaaaaagta aagaacatag gccccctggc
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5941 cgtctccgcc acgtctccc tctcacggcc gaccccgcag cccttttgcc ctttcctagg
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6061 gcccttgtgc tggtgatcgc ttggaagcgc atgcgaagac gaaggggagg agcagggcggc
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6241 gccgcttctt gtcgtcgttc cgagacatgt tagcagatcg cagtgccacc tttctgacg
6301 cgtcggcccc catattcgga cgcaattgtc atgttagca caattggagc aaatctggcg
6361 agcagtagg cttttaagtt gcaaggcag agagcaaaagt gggacgcggc gtgattattg
6421 gtatttacgc gacggcccgg cgcgttagcg gcccttcccc caggccaggg acgattatgt
6481 atcaatattg ttgcgttcgg gcaactcgtc gagggtcctt gcgggctggg gagggggatc
6541 tgggaattgg aggtacgacc gagatggctt gctcgggggg aggtttcctc gccgagcaag
6601 ccagggttag gtgttcgct cttgactcgt tgtgcattct aggaccccac tgctactcac
6661 aacaagcc CA

Appendix III: List of primers

-Cloning:

PCR amplification from pE. coli vector, NcoI→NdeI. Annealing temperature: 65°C.

5'primer:

5'-AAGAAGGAGATATACTATGGACGA-3'

5'-AAGAAGGAGATATAATATGGACGA-3'

5'-AAGAAGGAGATATCATATGGACGA-3'

3'primer:

3'-CCAACTCAGCTTCCTCTAGAT-3'

-Screening (PCR):

Primerset 1: annealing temperature: 55°C

5'NUD3'-5comp: 5'-TTCGGCTGCTTGTGGTGGTGGG-3' (T_m: 54°C)

3'psaDterm: 5'-GTACAGGCGGTCCAGCTGCTG-3' (T_m: 65.7°C)

Primerset 2: Annealing temperature: 55°C, elongation: 2minutes.

5'NUD3'-5comp: 5'-TTCGGCTGCTTGTGGTGGTGGG-3' (T_m: 54°C)

NUD3': 5'-GCTGGGGTAGTGCCGCATAGCCGC-3' (T_m: 71.3°C)

Primerset 3: Annealing temperature: 66°C

5'NUD GFP: 5'-CTGCCGCCCTGTGGCGTT-3' (T_m: 67.6°C)

3'psaD495: 5'-GCGAAAGCCTCCGAGCTCCGAT-3' (T_m: 65.8°C)

Primerset 4: Annealing temperature: 60°C

5'paro4331: 5'-ACGGCCGACCCGCCCCACGT-3' (T_m: 69.6°C)

3'paro4743: 5'-GATCCCGTACCTCGTGTGT-3' (T_m: 59.8°C)

Appendix IV: Solutions and recipes in (alphabetical order)

Work with DNA:

DNA gel loading buffer:

4% sucrose, 0.25% bromphenol blue.

TAE (Tris-acetate EDTA) buffer [50X]:

242g Tris base, 57.1mL glacial acetic acid, 100mL 0.5M EDTA pH 8.0 in 1L.

Work with *Chlamydomonas reinhardtii*:

-Media for growing C. reinhardtii

Phosphate stock [50X]:

47g $K_2HPO_4 \times 3H_2O$ (36g water free), 18g KH_2PO_4 in 500mL.

Salt stock [50X]:

12.50g NH_4Cl , 0.5g $MgSO_4 \times 7H_2O$, 0.25g $CaCl_2 \times 2H_2O$ in 500mL.

Hutner trace elements:

$Na_2EDTA \times 2H_2O$	5g
$ZnSO_4 \times 7H_2O$	2.2g
H_3BO_3	1.14g
$MnCl_2 \times 4H_2O$	506mg
$FeSO_4 \times 7H_2O$	499mg
$CoCl_2 \times 6H_2O$	161mg
$CuSO_4 \times 5H_2O$	157mg
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	110mg
dH ₂ O	100mL

Adjust pH to 6.5-6.8 with KOH (ca. 1.6g) at 70°C. Store at 5°C and wait until the solution has turned to a violet color before use.

-Isolation of chloroplasts

5X GR buffer:

2.23g $Na_2P_2O_7 \times 10H_2O$, 59.58g HEPES, 300.6g sorbitol, 20mL Na-EDTA ([0.5M], pH 8.0), 1.01g $MgCl_2 \times 6H_2O$, 0.99g $MnCl_2$. Dissolve pyrophosphate in 20mL boiling dH₂O, mix remaining ingredients in 750mL dH₂O. Combine solutions, adjust pH to 6.8 with 6N NaOH, and bring to 1L. Freeze in 20mL aliquotes.

PCBF:

Mix 17.5mL Percoll, 0.525g PEG 6000, 0.18g Ficoll, 0.18g BSA. Store at 4°C and use within 48 hours.

Isoascobate buffer:

0.595g HEPES, 4.4g isoascorbic acid in 40mL dH₂O. Adjust pH to 7.0 with 6M NaOH, dilute to 50mL and freeze in 1mL aliquots.

Mixing the discontinuous gradients:

	20% (v/v)	45% (v/v)	65% (v/v)
5X GR buffer	2.36 mL	2.36 mL	2.36 mL
Isoascorbate	0.13 mL	0.13 mL	0.13 mL
Glutathione	2.25 mg	2.25 mg	2.25 mg
PCBF	2.63 mL	5.90 mL	8.50 mL
dH ₂ O	8.00 mL	4.75 mL	2.11 mL

Mix the ingredients together and pipet 3 mL 65% solution to the bottom of a 15 mL corex centrifuge tube. Carefully pipet 3 mL 45% solution on top of the 65% solution by holding the corex tube at a 45° angle. Let the solution drip down along the tube wall. Repeat with 3 mL of 20% solution. The gradient can be stored for 1-2 days at 4°C. Avoid shaking.

Isolation buffer:

27.4g sorbitol, 5.96g HEPES-KOH ([50mM], pH 7.5), 2mL Na-EDTA ([0.5M], pH 8.0), 0.1g MgCl₂ x 6H₂O, 5.0g BSA in 490mL. Adjust pH to 7.5, dilute to 500mL. The buffer can be stored for two weeks.

Analytical methods:

SDS-PAGE:

Gel staining solution (SDS-PAGE):

Coomassie blue R-250 [0.1%], Methanol [46%], acetic acid [8%].

Gel destaining solution (SDS-PAGE):

Methanol [20%], acetic acid [5%].

SDS-PAGE running buffer [10X]:

30g Tris base, 146g glycine, 10g SDS in 1L.

SDS-PAGE sample buffer [3X]:

Tris-HCl [188mM, pH 6.8, SDS [6%], glycerol [30%], bromphenol blue [0.03%], β-mercaptoethanol [15%].

Solution A (SDS-PAGE):

29.2g acrylamide, 0.8g N',N'-bis-methylene-acrylamide in 100mL. Store in refrigerator in the dark.

Solution B (SDS-PAGE):

18.5g Tris base in 60mL dH₂O. Adjust pH to 8.8 with HCl. Add 4mL SDS [10%], 200μL TEMED (=N,N,N',N'-Tetramethylethylenediamine) and fill up to 100mL.

Solution B' (SDS-PAGE):

6.17g Tris base in 60mL dH₂O. Adjust pH to 6.8 with HCl. Add 4mL SDS [10%], 800μL TEMED (=N,N,N',N'-Tetramethylethylenediamine) and fill up to 100mL.

DNA and RNA blots:

Hybridization buffer:

50mL Na-phosphate [0.5M], 200 μ L EDTA [1mM], 1g BSA, 7g SDS in 100mL.

Labeling buffer (primer labeling):

First mix the following three solutions:

1) 625 μ L Tris [1M] pH 8.0, 62.5 μ L MgCl₂ [1 M], 8.7 μ L β -mercaptoethanol [14.4M] in 1mL.

2) 16.4 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in 25mL pH 6.6.

3) Random, primers (pd[N]₆) (Pharmacia), 50 units/mL in TE (Tris [10mM], Na₂-EDTA [1mM] pH 7.5.

Mix 475 μ L solution 1), 500 μ L solution 2) and 25 μ L solution 3).

Wash buffer # 1:

40mL Na-phosphate [40mM], 2mL EDTA [1mM], 5g BSA, 50g SDS in 1L.

Wash buffer #2:

160mL Na-phosphate [40mM], 8mL EDTA [1mM], 40g SDS in 4L.

-RNA blots:

MOPS buffer [5X]:

MgSO₄ [10mM], MOPS [0.5M], NaCl [2.5M]. Adjust pH to 7.5 with NaOH. Filter to sterilize and store the solution in the dark.

Na-phosphate buffer [1M, pH 7.2] (hybridization):

134g Na₂HPO₄ x 7H₂O, 4mL H₃PO₄ [85%] in 1L.

RNA gel loading buffer:

0.5mL glycerol [100%], 4 μ L Na₂-EDTA [250mM pH 8.0], 2.5mg bromphenolblue, 2.5mg xylene cyanol FF in 1mL. Treat with DEPC before use.

RNA transfer buffer:

NaOH [10mM], NaCl [3M].

-DNA blots:

Neutralization solution:

Tris [1.5M] pH 7.4, NaCl [1.5M].

Denaturation solution:

NaOH [0.5M], NaCl [1M]

SSC [20X]:

175.3g NaCl, 88.2g Sodium citrate x 2H₂O in dH₂O. Adjust pH to 7.0 with a 10N NaOH solution and bring to 1L.

Potassium acetate (5M potassium, 3M acetate):

Dissolve 294.42g potassium acetate in dH₂O. Adjust pH to 4.6 and bring to 1L.

TEG buffer:

Tris-HCl [25mM] H 8.0, Na₂-EDTA [10mM] and glucose [50mM] in 1L.

Western blot:

PBS buffer [10X]:

85g NaCl, 14g Na₂HPO₄, 2gNaH₂PO₄ in 1000mL. Adjust pH to 7.4.

Washing buffer Western blot:

0.5 tween 20 in 1000mL PBS buffer [1X].

Primary antibody dilution buffer:

1g BSA in 100mL Washing buffer. Adjust pH to 7.4.

Blocking buffer:

5g non-fat dry milk in 100mL PBS.

Transfer buffer western blot:

100mL SDS-PAGE electrode buffer [10X], 200mL methanol in 1000mL.

Appendix V: Endogenous *rppH* sequence from *C. reinhardtii*

The sequence can be found in the phytozome database:

<http://www.phytozome.net/genePage.php?search=1&detail=1&crowns&method=0&searchText=transcriptid%3A27580574>

5'UTR- red

Exons- purple

Introns- lower case letters

3'UTR- green

Our chosen start codon- grey background

>g4577

ACAGTACCCAGAAAACCTATCCCTGCCATCGCAAGCTTATAACCGCGTTTGTTGGTTCTTTCTTGACAACTAGG
GTCATCATCCCCTCCTGACCTGTTTGCCTGCGCGGCCGTCGCGAGGCTCTCACGTCAACATATACTTGCTTTT
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CGGCCGTCGCCATCCTCGAGCGACTACATAAACCCAGCGTACTTCTACGCCAACCCCTTCGCGCCAGCTCAGG
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TCGGTCTTTGCGGCCAGCGCAGCACGCGCCGAGCCACCGCCGCGCGGCCGTAGCGGCACGCTCTGCT
TCCCCTCCCCCTCCTCCTCGAGCGCTTCCAGCTCTCCCGCAGCCAGACGCACGCCCGTGCGCCGCGGTATC
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TGTCGCTGCAGCCGCTCCTGCGTAGCCACCAACACCTTGGCAGCATGGACGAGGGAGACGCGCCCCGCTGC
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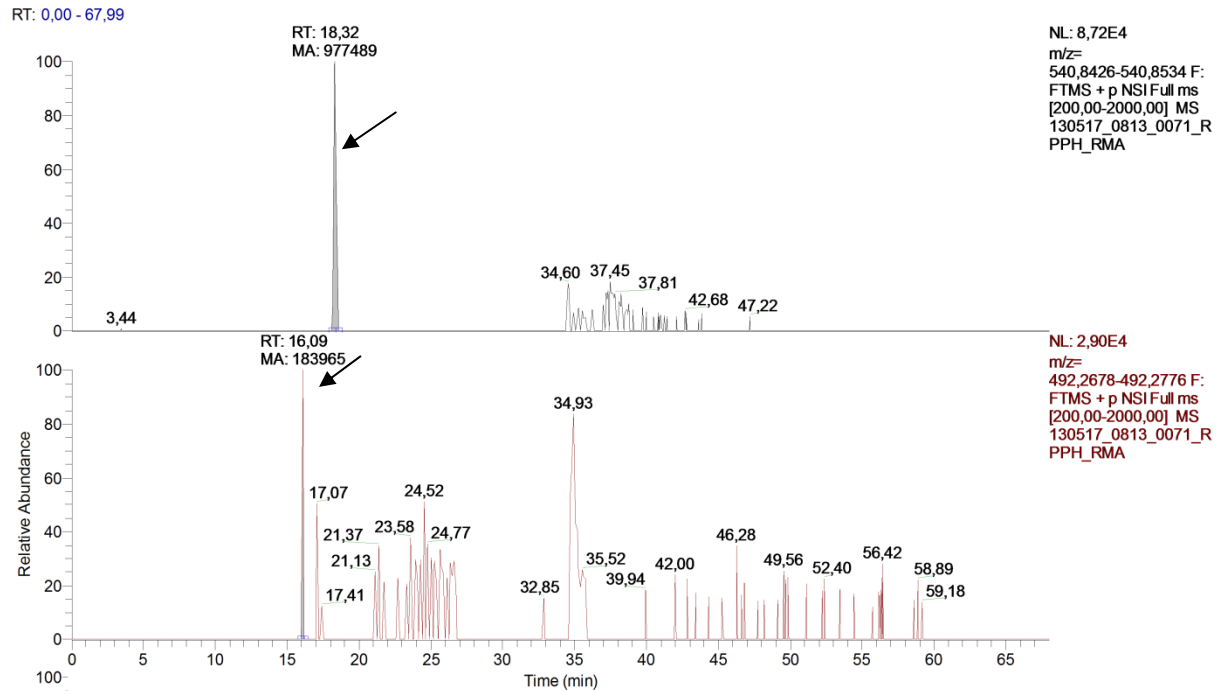
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Appendix VI: Mass spectrometry analysis

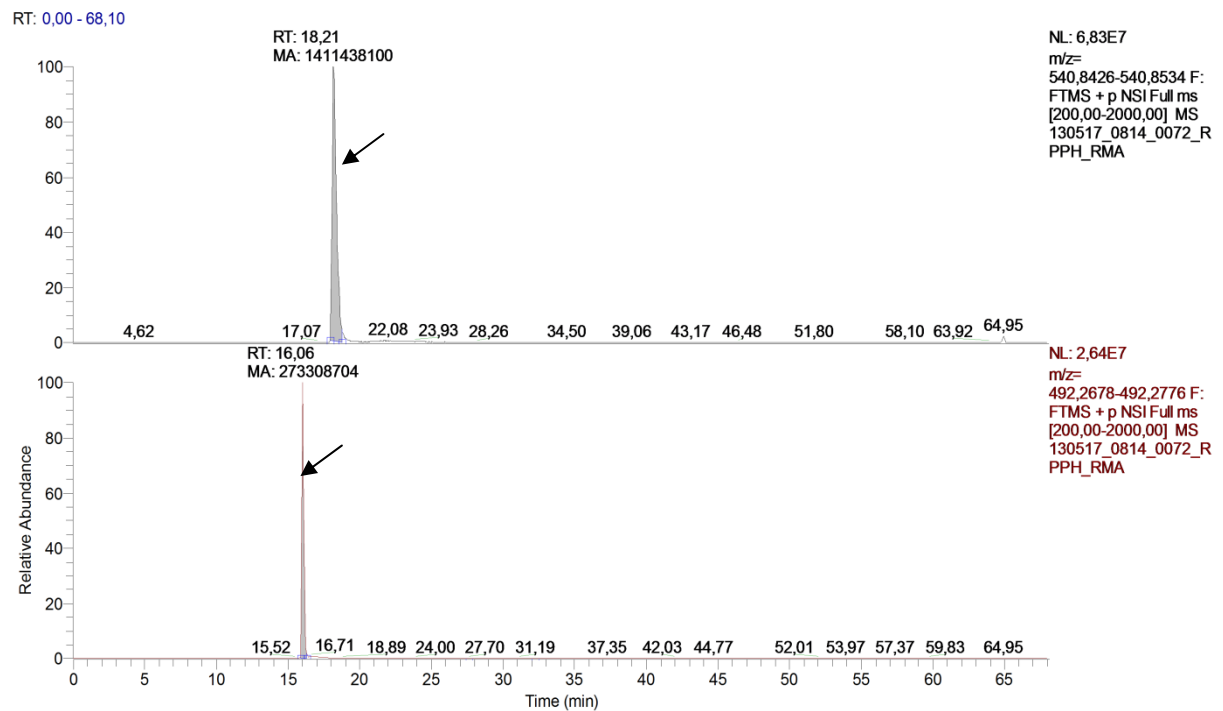
(The peaks corresponding to the specific peptides are shown by arrows.)

RppH-6xHN

Crude extract from *C. reinhardtii* transformant number four (sample)



Purified RppH-6xHN from *E. coli* (positive control)

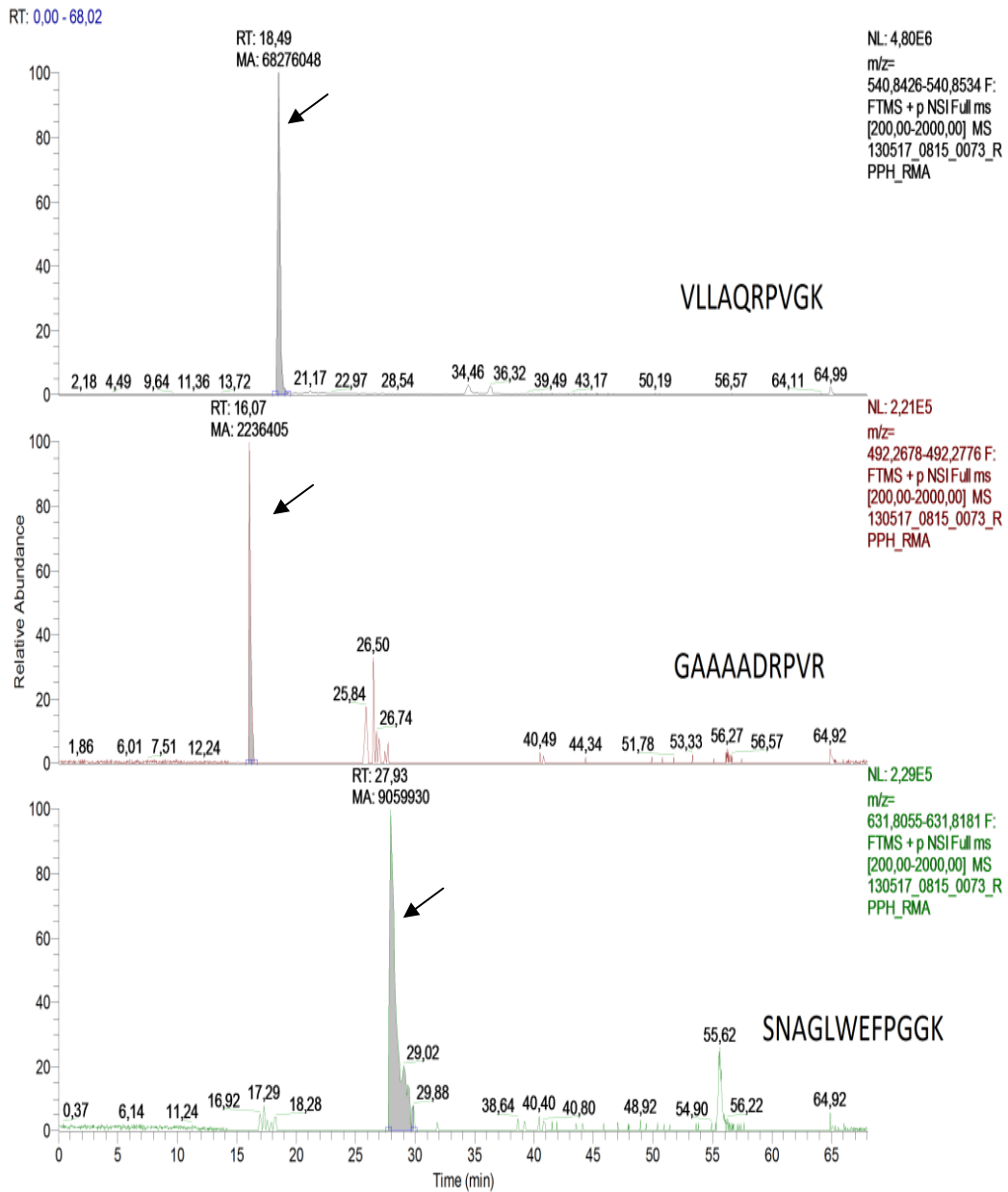


5'RppH-GFP-6xHN:

(The peaks corresponding to the specific peptides are shown by arrows. Fragmented peptides are shown as red peaks.)

Crude extract from *C. reinhardtii* GFP5 transformant (sample)

Not fragmented peptides:



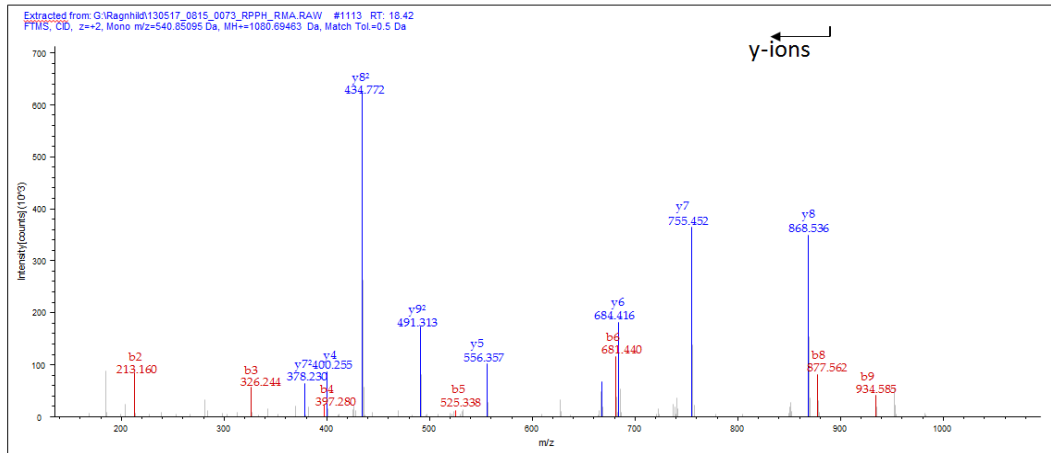
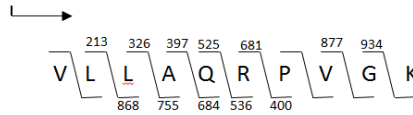
Fragmentated peptides:

VLLAQRPVGK

$[M + 1H]^+$
1080.6888

$[M + 2H]^{2+}$
540.8480

b-ions

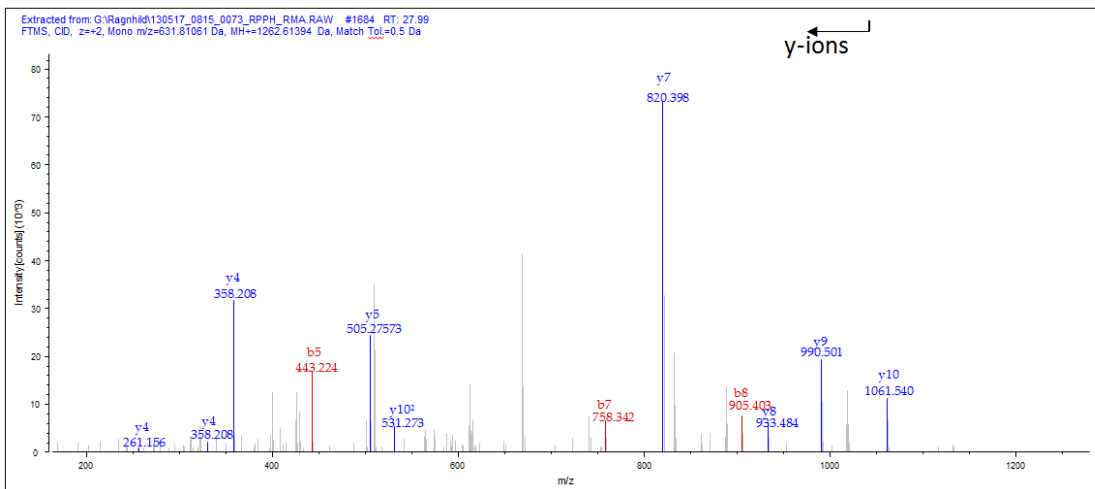
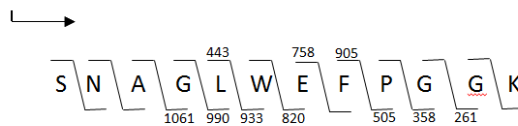


RPPH-GFP, SNAGLWEFPGGK

$[M + 1H]^+$
1262.6164

$[M + 2H]^{2+}$
631.8118

b-ions



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