



BIO – 3910

MASTER'S THESIS IN BIOLOGY

**Molecular biology and physiology of isolated chloroplasts
from the algae *Vaucheria***

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January 2010

Faculty of Biosciences, Fisheries and Economics

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University of Tromsø

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Acknowledgements

I'm very grateful to my supervisor, professor Kirsten Krause. It was her ability to cross the space between the knowledge levels and to bring closer some insights into the biological mysteries that actually initiated my commitment to fulfill a master degree in biology. Her guidance throughout the process has been most inspiring and educational.

I would very much like to thank all the people working at the plantphysiology and microbiology department at the University of Tromsø. I have always felt welcome to ask for any possible help and support that I needed. In this way I would like to express special regards to Janina Fuss, Ullrich Hermann, Hanne Risan Johansen and last, but not at all least, Bernd Ketelsen.

The initial cultures of *V. bursata* and *V. litorea* were kindly donated by laboratory of Prof. P. Kroth (Konstanz, Germany) and Prof. M. Rumpho (Orono, Maine, USA), respectively.

No thesis in my name would ever be possible without the support and tolerance of my husband and my children, who just accepted that mummy is writing a never ending homework, also during Christmas.

Abbreviations

³² P-CTP	Cytosine Triphosphate containing radioactive Phosphorus
³³ P-UTP	Guanosine Triphosphate containing radioactive Phosphorus
³⁵ S-met/cys	methionine and cystein containing radioactive Sulphur
ADP	Adenosine Diphosphate
ASW	Artificial Sea Water
ATP	Adenosine Triphosphate
BM	Basal Medium
BSA	Bovine Serum Albumin
Ci	Curie
cpDNA	chloroplast Deoxyribonucleic Acid
cpm	counts per minute
cpRNA	chloroplast Ribonucleic Acid
ddNTP	dideoxynucleotide Triphosphate
dH ₂ O	distilled water
dNTP	deoxynucleotide Triphosphate
dsDNA	double stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
EtOH	Ethanol
GC	Guanosine Cytosine
LB	Luria Bertani
MCS	Multiple Cloning Site
MOPS	4-Morpholinepropanesulfonic Acid
mRNA	messenger Ribonucleic Acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	reduced Nicotinamide Adenine Dinucleotide Phosphate
NEP	Nuclear-Encoded RNA Polymerase
PCR	Polymerase Chain Reaction
PEP	Plastid-Encoded RNA Polymerase
P _i	Inorganic phosphate
PS	Photosystem

rcf	relative centrifugal force
RER	rough endoplasmic reticulum
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Standard Sodium Citrate
ssDNA	single stranded Deoxyribonucleic Acid
SSU rDNA	Small Subunit ribosomal DNA
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TMV	Tobacco Mosaic Virus
tRNA	transfer Ribonucleic Acid
UTR	untranslated region
UV	Ultraviolet

Abstract

Sea slugs of the genus *Elysia* (e.g. *E. chlorotica*) are known for their ability to incorporate chloroplasts from the yellow-green alga *Vaucheria litorea*. These “kleptoplasts” stay active in the digestive tract of the sea slug for several months. Chloroplasts from *Vaucheria litorea* are also reported to be significantly more stable after *in vitro* isolation than chloroplasts of other algae or of higher plants. *In organello* assays with isolated chloroplasts are used in studies on photosynthetic and biochemical processes in these organelles, chloroplast-nucleus communication, plant development and plant responses to environmental changes. The major limiting factor of the chloroplast *in organello* systems is the rapid decline in function and intactness of the plastids. This study is investigating the stability and longevity of chloroplasts isolated from *V. litorea* and its relative *V. bursata* in comparison to an angiosperm *Pisum sativum*. The structural intactness of the chloroplasts was investigated by phase contrast microscopy and the overall transcriptional activities were analyzed by run-on transcription assays. The recently completed sequence of the plastid genome from *V. litorea* has made it possible to investigate also the specific changes in isolated chloroplasts on the transcriptional level. The expression patterns of chloroplast-encoded genes *trnE*, *rrn23*, *rrn16*, *rbcL*, *psbD*, *psbA* and *psaA* directly after isolation and 4 hours post-isolation time were analyzed using dot blot hybridization. The ability of isolated chloroplasts to incorporate ³⁵S-methionine into *de novo* synthesized proteins at 0, 4 and 24 hours after isolation was tested by translational assays. The chloroplasts of *Vaucheria litorea* were both transcriptionally and translationally stable over an extended period of time. However, the culturing limitations and low plastid yields diminish the potential of this alga as a chloroplast donor for *in organello* assays. Chloroplasts of *Pisum sativum*, previously suspected to be more unstable, did not show a decline in transcription rate until 4 hours after isolation. The decrease in incorporation of ³⁵S-methionine between time 0 and 4 hours after isolation was only minor, between 4 and 24 hours more significant but the radioactive signal was still readily detectable at 24 hours. *Pisum sativum* has simple growth requirements and offers high yields of isolated chloroplasts. Chloroplasts from this plant should therefore be in the centre of further investigations into their suitability to express foreign genes and their potential for *in organello* assays.

1. Introduction

Virtually all life of higher form is dependent on oxygen (Nelson and Ben-Shem, 2004) and most of the atmospheric oxygen is produced by land plants as by-product of photosynthesis in organelles called chloroplasts. During photosynthesis, specialized protein complexes, photosystems PSI and PSII, which are embedded in internal chloroplast membranes, absorb photons and transfer their energy to chlorophyll, their core pigment molecule. When enough energy is captured, a high energy state electron is released from chlorophylls and shuttled through protein complexes of the electron transport chain where the energy is used to generate a hydrogen proton gradient across the thylakoid membrane. The resulting directional protonation of ATP synthase complex, also traversing the thylakoid membrane, powers the phosphorylation of ADP while dissipating the proton gradient. The released electrons are ultimately used to reduce NADP to NADPH, a molecule that provides energetic electrons and protons for other processes in the chloroplast. The electrons and protons consumed in the light reaction are replenished from splitting water to hydrogen ions and O₂. The chemical energy that has been conserved in form of ATP and NADPH is then used for the light-independent assimilation of CO₂ by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the synthesis of carbohydrates (Berg et al., 2006).

Besides the production of energy and carbohydrates just from light and water, the chloroplasts also contribute to other metabolic processes such as synthesis of amino acids, fatty acids, the lipid components of their own membranes, vitamins and plant hormone precursors. The reduction of nitrite (NO₂⁻) to ammonia (NH₃), an essential step in the incorporation of nitrogen into organic compounds, also occurs in chloroplasts (Cooper and Housman, 2000).

1.1 The chloroplast genome

Already in 1909 the German botanist Erwin Bauer made the observation that leaves of *Pelargonium* and *Mirabilis* did not follow Mendelian inheritance and that the characters of inheritance were associated with chloroplasts. The real proof that chloroplasts really do possess their own DNA came in 1963 when Ruth Sager and Masahiro R. Ishida detected DNA with GC content distinguishable from nuclear DNA in relatively intact chloroplasts isolated from the green algae of the genus *Chlamydomonas* (Sugiura M., 2002).

The chloroplast genome (cpDNA) is a circular double stranded DNA molecule of a size of 120 to 160 kb, with few exceptions among green algae (*Codium fragile* 85kb, *Acetabularia* 2000kb) (Sugiura, 1992). The common feature is presence of inverted repeat sequences (IR) with sizes between 6 – 76kb which usually contain the rRNA gene cluster and in some species also some additional genes. The IR is separated by one large and one short single copy DNA sequence. In some species the IR can be tandemly repeated as in the unicellular protist *Euglena gracilis* that has three tandem repeats. The size difference in most chloroplasts is due to the size difference and the copy number of the IR (fig.1).

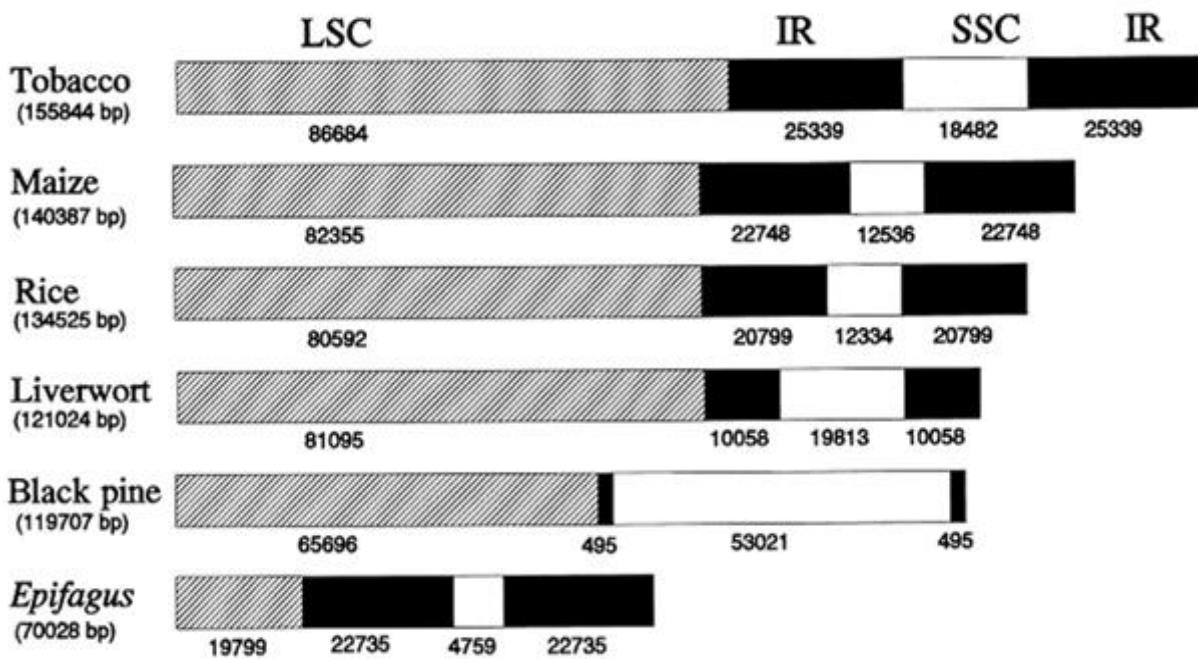


Figure 1: Size comparison of some sequenced genomes from chloroplasts of higher plants. IR (filled boxes) – inverted repeat regions, LSC (boxes with striations) – large single copy region, SSC (open boxes) – small single copy region (Maier et al., 1995)

In average, the chloroplast genome codes for 120 genes, most of them being arranged in 50 clusters called operons. Genes within each operon are under the same regulation and are transcribed as a unit. Their products are usually, but not always, involved in the same organellar process. The presence of introns (transcribed but not translated intragenic sequences) within operons also varies from species to species. About half of the chloroplast genes code for proteins and RNA molecules involved in gene expression in the organelle. The chloroplasts contain ribosomes with a sedimentation coefficient of 70S that are distinct from cytoplasmic 80S ribosomes. The chloroplast genome codes for all ribosomal RNAs, approximately one third of ribosomal proteins, all 20 – 40 transfer RNAs and subunits of a

RNA polymerase. The remaining 40 – 60 genes code for large subunits of the PSI and PSII, electron transport chain complex Cytochrome b_6/f , ATP synthase and Rubisco and some genes of yet unknown function (ycf genes) (Leister 2003; Sugiura 1992).

1.2 Protein import and chloroplast-nucleus communication

While chloroplast genomes of most species code for only up to a few hundred proteins it was estimated for the model organism *A. thaliana* that chloroplasts need 2100 – 3600 proteins for biogenesis and function (Leister 2003). This means that more than 90% of the plastid proteins are encoded by nuclear DNA. The precursor peptides of chloroplast proteins are synthesized on free cytosolic ribosomes with amino-terminal chloroplast targeting transit sequences and transported into chloroplasts post-translationally. In the chloroplast stroma, the transit sequence is cleaved off by a stromal metalloendopeptidase specific for imported peptides, yielding the mature protein. Proteins targeted further to the thylakoids have additional transfer sequences that are also cleaved off by a specific thylakoid peptidase (Bassham et al., 1991).

The dependence of chloroplasts on import of nucleus encoded proteins and the reciprocal dependence of the cell on metabolites produced by the chloroplasts require that nucleus-chloroplast communication and signaling is going in both directions. Both plastid and nuclear encoded subunits of the photosynthetic complexes are present in the organelle in the correct ratio required for the assembly. With several hundred copies of chloroplast DNA in the cell and mostly only two copies of nuclear DNA, the synthesis must be synchronized to avoid unnecessary overproduction of chloroplast encoded subunits and to ensure enough protein import into the organelle when needed (Choquet and Wollman, 2002).

The chloroplast gene expression is directly regulated by the nucleus. The genes coding for the multi-subunit plastid-encoded RNA polymerase (PEP), transcribing genes for photosynthesis, are transcribed by a nuclear single subunit RNA polymerase (NEP). The PEP enzyme also requires nucleus encoded sigma subunits for specific promoter recognition and possibly additional nuclear transcription factors. At early stages of chloroplast development, before the establishment of PEP, the organelle is entirely dependent on the nucleus (Brautigam et al., 2007).

Another example of the nucleus' involvement in chloroplast gene expression is the translation of mRNAs for photosynthetic subunits, as for example PsbA, the core protein subunit of PSII.

The translation of certain mRNAs requires binding of a nucleus encoded translation activator complex to the 5' untranslated region (5'-UTR). This activation is, however, originally initiated by the chloroplast itself. In light, the complex can bind to *psbA* mRNA because the redox-responsive regulatory site of the complex is reduced by the NADPH dependent reducing agent thioredoxin. In dark, on the other hand, the regulatory site is phosphorylated in an ADP-dependent reaction due to high levels of ADP (Trebitch et al., 2000).

The influence of chloroplasts on gene expression in nucleus was investigated in Arabidopsis using genomes uncoupled (GUN) mutants. When compared to wild type, the expression of *Lhcb*, nuclear gene coding for the chlorophyll a/b binding protein of PSII, was not downregulated in plants where chloroplasts were destroyed by inhibition of carotenoids that protect the proteins of light-harvesting complexes from photodamage. It was discovered that Mg-protoporphyrin IX, the product of Mg-chelatase reaction that takes place in chloroplasts, accumulates inside the organelle in the dark and is somehow released in a light dependent manner and switches the expression of chloroplast nuclear-encoded genes on and off (Jarvis 2003).

1.3 Involvement of chloroplasts in cell death

Despite the evident function of chloroplasts as vital energy and metabolite production centers, recent research has showed their involvement also in light independent plant processes such as response to infections and programmed cell death (PCD) in monocarpic (dying after seed production) plants (Doorn and Yoshimoto, 2009). Young seeds of monocarpic plants produce chemicals that induce yellowing, followed shortly after by death of the whole plant. Deactivation of NdhF, a chloroplast-encoded subunit of the NADH dehydrogenase complex involved in PSI electron transport that is essential for proton gradient regulation, altered flowering and yellowing in tobacco plants. Transgenic tobacco plants with a knockout of the *ndhF* gene started to flower ten days earlier than wild type plants and did not show any leaf yellowing until late stages of fruit development. In wild type tobacco plants, the onset of flowering triggers almost immediate yellowing, progressing from basal to apical leaves (Doorn and Yoshimoto, 2009).

Seo et al. (2000) reported the role of another chloroplast protein, DS9, connected with the response of tobacco plants to tobacco mosaic virus (TMV) infection. The DS9 protein is homologous to bacterial FtsH metalloprotease and is most likely involved in degradation of

damaged and unfunctional chloroplast proteins. Its gene is constitutively transcribed in high levels in tobacco plants resistant to TMV. After infection, the levels of DS9 in chloroplasts decline abruptly which in return accelerates the hypersensitive reaction of the cell. The infected cell dies before the virus can spread to the neighboring cells (Doorn and Yoshimoto, 2009; Seo et al., 2000). Further research on chloroplast regulation and chloroplast-nucleus communication will be required to understand the depth of the influence of these organelles on plant growth.

1.4 Endosymbiotic origin of chloroplasts

Even though the main oxygen producers at present time are land plants and algae, the pioneers responsible for the evolution of an oxygenic atmosphere at the dawn of life were organisms resembling modern cyanobacteria (Schopf, 1993). It is now a commonly accepted knowledge that chloroplasts actually originate from these photosynthetic bacteria. One of the first quantitative evidences supporting the endosymbiotic theory has come as late as in 1975 by Linda Bonen and W. Ford Doolittle. With a rudimentary Sanger-sequencing method they showed extensive homology between 16S rRNA of the small ribosomal subunit of *Porphyridium* (a unicellular marine red alga) with 16S rRNA of *E. coli*, *B. subtilis* and *A. nidulans* (a blue-green alga). They also showed that there is no homology of organellar 16S rRNA sequence with the cytoplasmic 18S rRNA of *Porphyridium*, suggesting that the chloroplasts are more closely related to even non-photosynthetic bacteria than the organism in which they reside.

Even though the various chloroplasts in the organisms from major eukaryotic superclusters as Plantae, Chromalveolate, Rhizaria and Excavata (Gould et al., 2008) are believed to share a common cyanobacterial ancestor (Cavalier-Smith, 2000; Sandelius and Aronsson, 2009), the acquisition of chloroplasts through the process of engulfment and subsequent retention of the functional organelle in the host cell has occurred independently more than once. Many chloroplast containing organisms have organelles surrounded by multiple membranes. These structures could only arise by engulfment of a eukaryote by another eukaryote and a reduction of everything but the primary chloroplast of the absorbed organism (Ludwig and Gibbs, 1985). The number of membranes was previously used as an indication of the number of endosymbiotic events. The engulfed cyanobacterium was contained in the food vacuole creating an additional membrane of the host's origin. The bacterial peptidoglycan cell wall was eventually lost in all lineages except for glaucophytes (Cavalier-Smith, 2002). The

secondary symbiosis would result in four membranes and other symbiotic events would further contribute with an additional double membrane. However, the number of membranes can be misleading as one or more membranes have also been lost secondarily in some cases during the evolution. Examples are dinoflagellates with secondary but only double-membraned chloroplasts or triple-membraned chloroplasts of euglenoids (fig. 3) (Cavalier-Smith, 2000; Sandelius and Aronsson, 2009). In those algae that have secondary chloroplasts with four membranes, the outermost membrane is continuous with the membrane of rough endoplasmic reticulum (RER). The ribosomes are attached to RER membrane on the cytosolic, opposite side than the chloroplasts which indicates that the organelles actually lie within the lumen of RER (fig. 2) (Chaal and Green, 2005).

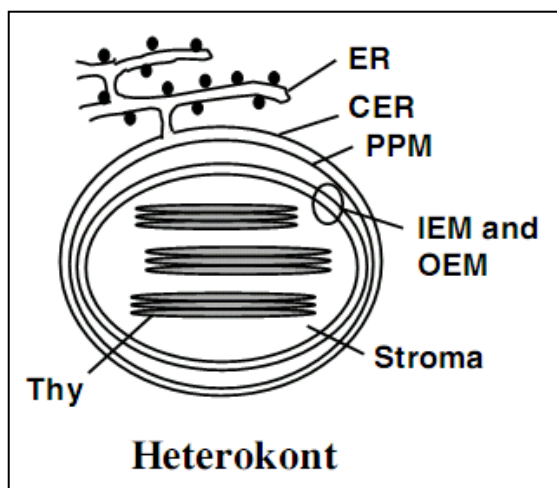


Figure 2: The chloroplast of heterokont alga surrounded by four membranes. The outer most membrane, referred to as chloroplast endoplasmic reticulum (CER), is continuous with rough endoplasmic reticulum (RER). IEM – inner envelope membrane, OEM – outer envelope membrane, PPM - periplastid membrane, ER – endoplasmic reticulum (Chaal and Green, 2005)

During the establishment of a functional organelle, a more radical reduction of the endosymbiotic organism led in some cases to the loss of the photosynthetic ability. Several members of ciliates, dinoflagellates, heterokontophytes, apicomplexans and trypanosomatids possess nonphotosynthetic plastids originating from cyanobacteria but still providing the cells with essential metabolic pathways such as fatty acid synthesis and isoprenoid synthesis as in the case of malaria parasites (Gould et al., 2008).

The events that resulted in the establishment of primary and secondary chloroplasts as described in this section occurred during the evolution of present species many million years ago. An endosymbiotic uptake of chloroplasts into organisms previously known as obligate heterotrophs has been discovered rather recently and will be described in the next chapter.

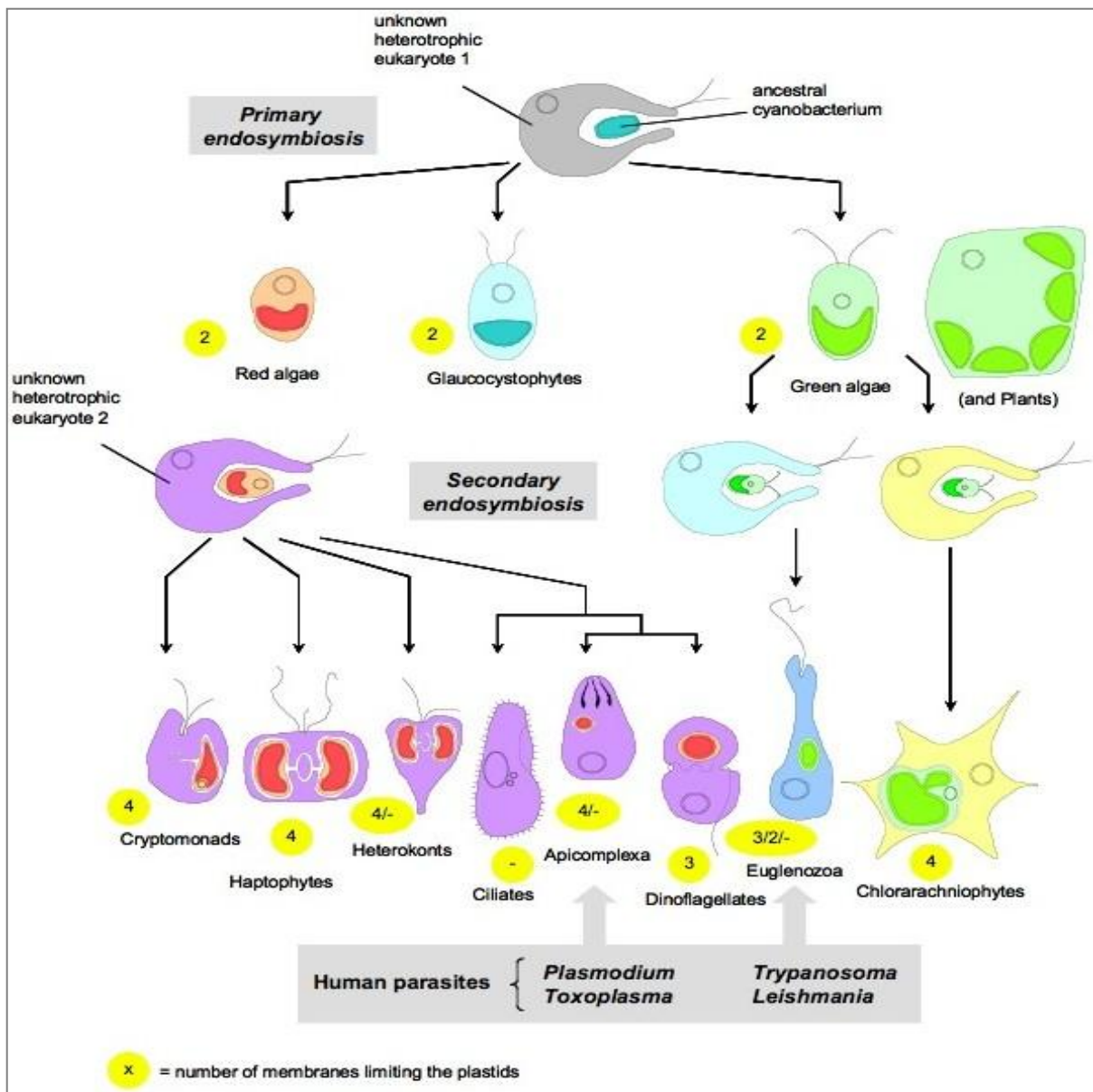


Figure 3: Origin and evolution of plastids by primary and secondary endosymbioses. Single primary endosymbiosis between an unknown heterotrophic eukaryote and a Gram-negative cyanobacterium led to the three primary plastid-bearing lineages, Green algae and plants, Glaucocystophytes and Red algae. The primary plastid is always surrounded by an envelope consisting of two membranes. Two types of secondary endosymbiosis involving two different green algae and unrelated unknown heterotrophic eukaryotes led to a development of Euglenozoa and Chlorarachniophytes. A single endosymbiosis between a red alga and a heterotrophic eukaryote led to all remaining plastid bearing protists. Loss of photosynthesis occurred in several lineages and in ciliates, the plastid was apparently lost in the entire phylum. The number of membranes limiting primary and secondary plastids is highlighted in yellow. Unicellular parasites causing major human threats are highlighted in grey. In the case of Trypanosomatidae, the plastid was lost in the complete phylum. In the case of Apicomplexa, the plastid was lost in some genera such as *Cryptosporidium* and conserved in others such as *Plasmodium* or *Toxoplasma*. Adapted from Archibald and Keeling (2002).

1.5 “Kleptoplasts” in the sea slug *Elysia chlorotica*

As early as in 1876 De Negri and De Negri observed green pigment in the sea slug *Elysia viridis*. In 1883 Brandt isolated these small green “bodies” from the slug (Rumpho et al., 2000), but they were left unstudied until as late as the end of the 1960’s. These green bodies were first identified as photosynthetically active algal chloroplasts in a marine slug *Elysia atroviridis* in 1965 by Kawaguti and Yamasu. Since then the presence of functional algal chloroplasts was reported in diverse organisms such as several different species of sacoglossan molluscs, marine ciliates, several families of Foraminifera and a Rotifer (Rumpho et al., 2007; Taylor, 1970). The ability of some animals to retain photosynthetically active chloroplasts free from any additional plant cell constituents or within their bodies while using the produced energy and carbohydrates is referred to as kleptoplasty, emphasizing the beneficial outcome for the host only (Rumpho et al., 2007).

One of the kleptoplastic organisms studied in more detail is *Elysia chlorotica*. *Elysia chlorotica* is a marine sacoglossan (“sap-sucking”) herbivorous mollusc found mostly in brackish salt marshes along the east coast of USA. It can be up to 6 cm long and even though it is a hermaphrodite it is usually cross-fertilized. After hatching at the late spring the planctonic larva feed for two weeks on unicellular algae. In laboratory experiments it was shown that the juvenile slug has to further feed specifically on the filaments of the coenocytic chromophyte *Vaucheria litorea* in order to develop into a mature adult animal. It ruptures the algae and sucks in the content of the filament, but only the chloroplasts are phagocytosed into the epithelial cells of the digestive tract. In the digestive cells the chloroplasts “escape” the food vesicle and are in direct contact with the cytosol (Rumpho et al., 2001). The chloroplasts remain active inside the animal. After sequestering substantial amount of chloroplasts within the branched digestive system just one cell layer beneath the epidermis, the photosynthate produced by these “stolen” organelles can support the growth of the sea slug, if provided with light. The slugs can survive without any additional food source for up to ten months. Besides a carbon source, the chloroplasts also provide precursors for chemical defenses, protective mucus and, through the change in morphology, leaf-resembling camouflage (fig. 4) (Rumpho et al., 2000). Even though there are various associations of different sacoglossan slugs with chloroplasts of other algal species, the chloroplast functionality in *E. chlorotica* is of exceptionally long duration (Pierce et al., 1999).



Figure 4: Images of adult *E. chlorotica* showing the characteristic green coloration and morphology

The life cycle of *E. chlorotica* is annual and all the population hatched one spring dies nearly simultaneously through the process of apoptosis the next spring (Mondy and Pierce, 2002). It has been observed that the death of the slugs both in their natural environment and in laboratory conditions is connected with the presence of retroviruses in the digestive cells of the animals. Retroviruses are inherited in a Mendelian manner as they establish as provirus DNA in the host genome. What exactly triggers the expression of the virus genes at the end of the animals' lives is not yet known, but in natural environment, the onset of the retroviral activity is coincident with the increase in the water temperature (Pierce et al., 1999).

1.6 The alga *Vaucheria*

1.6.1 Habitat and phylogeny

Taxonomy (www.algaebase.org)

Empire – Eukaryota, Kingdom – Chromista, Subkingdom – Chromobiota, Infrakingdom – Heterokonta, Phylum – Heterokontophyta, Class – Xantophyceae, Order – Vaucheriales, Family – Vaucheriaceae, Genus – *Vaucheria*, Species – *litorea*

There are over 70 known members of the genus *Vaucheria*. *Vaucheria* species are yellow-green, siphonaceous algae that occur widely spread around the world. Even though most of the species are euryhalinic and can acclimate from fresh water to full strength sea water, only few *Vaucheria*s are recognized as truly marine species. They are important desiccation-tolerant mud-stabilizers preventing corrosion by holding exposed soil with the submerged filaments.

The filaments are coenocytic, meaning that the cross-walls are formed only in reproductive cells or in case of injury. The large central vacuole is surrounded by cytoplasm containing spheroid chloroplasts and small multiple nuclei (www.algaebase.org). Reproduction is mostly either vegetative by simple fragmentation of the filaments or asexual involving production of various types of spores. The non-motile thin walled aplanospores are formed readily by septum formation at the tips of the filaments and are often used as a fast means of “escape” in unfavorable conditions. The tips of the filaments can also differentiate into zoosporangia producing a single motile multiflagellate, multinucleate synzoospore. Thick walled akinetes – separated portions of the filaments, are formed in low and dry conditions. Sexual reproduction involves formation of oogonia and antheridia with flagellate sperms (Sharma, 1986).

The taxonomy of *Vaucheria* species was traditionally based on morphology of the vegetative and sexual reproductive structures as oogonia and atheridia (Schagerl and Kerschbaumer, 2007). Unfortunately *Vaucheria* species in their natural habitat only rarely produce reproductive structures and therefore the knowledge of their ecology and biogeography on the species level is limited. This impairs other areas of research, such as the investigations of composition of algal populations and the changes within them in response to the changes in environment (Linne von Berg and Kowallik, 1996). Recent molecular studies of the chloroplast genome RFLPs and analysis of the *rbcL* coding regions as well as the spacer regions between *rbcL* and *psbA* genes are sometimes not consistent with previous morphological species identifications. It was suggested that chloroplast DNA, being only maternally inherited, may not provide the ideal molecular tool and analysis of nuclear DNA and more detailed morphological studies will be needed to systematically annotate the genus *Vaucheria* (Andersen and Bailey, 2002). However, *rbcL* sequences together with sequences for small ribosomal subunits (SSU rDNA) proved to be useful as molecular markers to distinguish *Vaucheriales* as a distinct order in a class *Xantophyceae*, including mostly unicellular and colonial algae, but also several multinucleate siphonous forms and multicellular filamentous species (Negrisolo et al., 2004). *Vaucheria litorea* was first described and appointed as a specific species by a Swedish botanist Carl Adolph Agardh in 1821.

1.6.2 Characteristics of *Vaucheria* chloroplast

The chloroplasts of *Vaucheria litorea* evolved from a secondary symbiosis of a eukaryotic organism absorbing a red alga. This ancestor organism replaced phycobillines of red algae with an accessory pigment chlorophyll c and gave origin to the three groups of chromista – heterokonts, cryptophytes and haptophytes, but also to chloroplasts of alveolata (ciliates, dinoflagellates and apicomplexa) (fig. 3) (Whatley and Whatley, 1981; Voolstra et al., 2009). As a remnant of the second endosymbiotic event, the chloroplasts of *V. litorea* inside the alga are surrounded by four membranes – inner double membrane, the standard chloroplast envelope present in all chloroplasts, a periplastic membrane around the envelope that is believed to be the plasma membrane of the red alga and a chloroplast endoplasmic reticulum (chloroplast ER) (Rumpho et al., 2001). Ribosomes associated with chloroplast ER translate nuclear-encoded mRNA coding for plastid proteins. These proteins are then translocated across the chloroplasts ER during synthesis and further transported to the chloroplast envelope in vesicles formed by the periplastic membrane (Bourne and Danielli, 1981). The chloroplast ER and periplastic membrane are present neither in the kleptoplasts of *E. chlorotica* nor in chloroplasts mechanically isolated from the alga (Rumpho et al., 2001).

1.6.3 The genome of *Vaucheria* chloroplasts

The chloroplast genome of *Vaucheria litorea* has been recently sequenced by Rumpho et al. (2008). It consists of a circular and double stranded DNA molecule, 115,341-bp long and codes for 169 genes (139 protein-coding genes, 27 tRNA genes and 3 rRNA genes) (fig. 5). The GC content is only 28% which is generally rather low, also in comparison with other heterokonts. Similar to other chloroplast genomes, the chloroplast is divided into one larger (62 002bp) and one smaller (43 469bp) single-copy region by an inverted repeat (see chapter 1.1). This IR is short (4 935bp) and both copies contain the highly conserved operon with all plastidic ribosomal RNAs (rRNAs). The *V. litorea* chloroplast genome contains one intron in the *trnL* gene coding for transfer RNA specific for leucine, a feature preserved mainly in the primary chloroplasts (James and Schmidt, 2004) from its cyanobacterial origin. It has also retained genes for light-independent chlorophyll synthesis which were not found in other heterokont genomes sequenced so far. However, despite these peculiarities, the cpDNA of *V. litorea* has a larger coding capacity than green plastid genomes and shares more similarity with other heterokonts and red algae than with plastids of the green lineage (Rumpho et al., 2008; Hagopian et al., 2004).

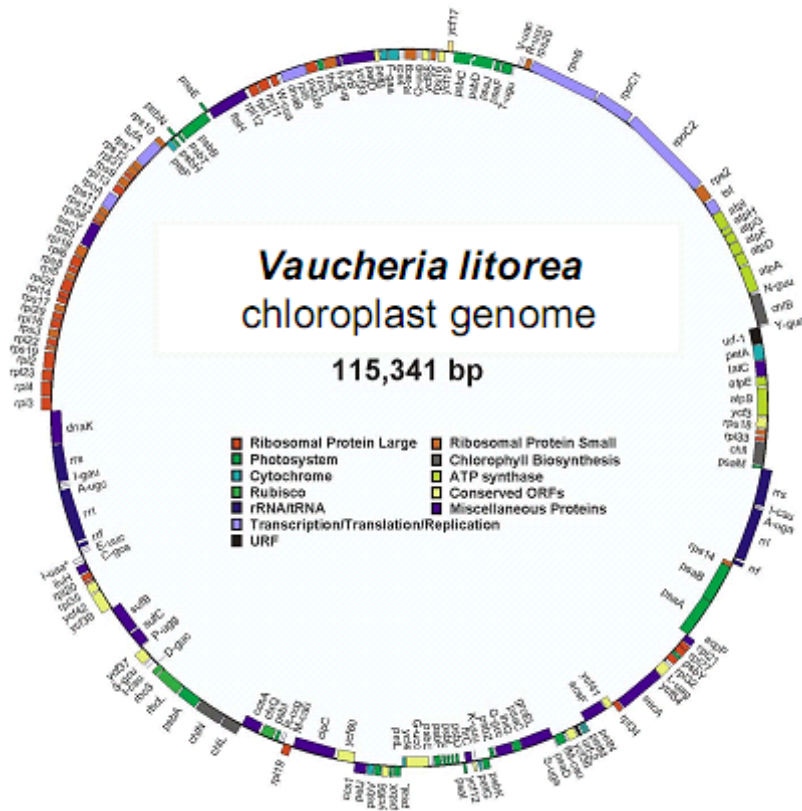


Figure 5: Map of the *Vaucheria litorea* chloroplast genome. Genes annotated on the outside of the DNA representation are transcribed in the clockwise direction, genes of the inside are transcribed in the counterclockwise direction. Genes are color coded according to their function as shown (Rumpho et al., 2008).

1.6.4 Plastid stability in the animal host

The ability of the otherwise typical secondary red algal chloroplasts of *V. litorea* to stay active in *E. chlorotica*, without access to nucleus encoded proteins, has raised the question of specific interaction between the organelle and the host organism. The nuclear-encoded gene for PsbO, the major core protein of the oxygen evolving complex PSII, and *fcp*, *Lhcv1* and *Lhcv*, other chloroplast nuclear-encoded genes, were detected in the nuclear DNA of the sea slug eggs that had never been exposed to the alga *V. litorea* (Pierce et al., 2007; Rumpho et al., 2008). The sequences of these genes in *E. chlorotica* were identical to the genes in cpDNA of *V. litorea*. The *psbO* gene also contained the tripartite targeting sequence necessary for protein import into secondary chloroplasts surrounded also by chloroplast ER. The presence of these genes in *E. chlorotica*, otherwise never found in any non-photosynthetic organism, suggests that they have been acquired through horizontal gene transfer (HGT) between nuclear DNA of *V. litorea* and nuclear DNA of *E. chlorotica* (Rumpho et al., 2008). The high sequence identity of these genes from the animal and from

the algae implies that the transfer has, from the evolutionary point of view, occurred only recently. As mentioned in chapter 1.5, the retrovirus responsible for the onset death of all the animals from the same generation could originate from the alga. Retroviruses are mobile genetic elements known to be able to transfer DNA sequences, neighboring the sites where the provirus was integrated in the first host genome, to another host genome. This virus was also present in laboratory animals that had never contact with any contaminants suggesting that it is passed on to the new progeny in a Mendelian manner. However, the insertion of provirus DNA into the host DNA is usually random. The acquisition of several photosynthetic genes necessary for stable chloroplast function in the sea slug would require numerous HGT events. Further, the assistance of viruses in gene transfer between *E. chlorotica* and *V. litorea* has not been verified yet (Rumpho et al., 2008).

Another possibility how to functionally stabilize the chloroplasts in *E. chlorotica* is through the action of nuclear-encoded mitochondrial proteins. There are proteins that have the same function both in chloroplasts and mitochondria, e. g. protoporphyrinogen oxidase II in spinach. This enzyme is necessary for biosynthesis of chlorophyll in chloroplasts and of haem in mitochondria and in chloroplasts. The mRNA for this protein has two in-frame translation initiation codons resulting in two proteins with only one targeting sequence. The longer protein product is imported into chloroplasts and the shorter into mitochondria. Another dual-targeting system, known to be harnessed by more numerous dual targeted proteins, uses single but ambiguous targeting sequence. The common feature is that they have only few negatively charged residues and they are enriched in arginine and serine. Majority of these ambiguously targeted proteins are involved in the organellar gene expression, but some also in protection against oxidative stress and other processes (Peeters and Small, 2001).

1.6.5 Plastid stability *in vitro*

Interestingly, chloroplasts isolated from *Vaucheria litorea in vitro* also show significant stability compared to chloroplasts of wheat, tobacco or spinach (Green et al., 2005). Traditionally measured O₂ evolution, CO₂ incorporation but also protein translation in isolated chloroplasts from higher plants is known to drop quickly after isolation, depending also on the isolation conditions, and usually cease completely during the first hour (Polanska et al., 2004; Nivison and Jagendorf 1984). The experiments done by Green et al. (2005) show that isolated chloroplasts from *Vaucheria litorea* are fairly robust and survive at high rate both the mechanical procedure and various media conditions during isolation. They also show

remarkable structural stability, even after 14 days of incubation, measured by phase contrast microscopy. The FeCN-dependent O₂ evolution and translational activity declined only by a few percent in 48 hours after isolation, but decreased significantly in comparison to phase contrast microscopy results after 72 hours post-isolation time. This could be explained by a loss of stromal metabolites due to disruption in the chloroplast envelope and subsequent resealing of the membranes. The incorporation of radioactive CO₂ dropped more dramatically during first 24 hours and then declined more slowly until 72 hours after isolation, but remained still as high as 20% of the activity measured at time 0.

1.7 Goals of the master thesis

As mentioned previously, the relationship between chloroplasts and their host cell can be characterized as mutualistic dependency. As the plant is dependent on chloroplasts for energy which is of course necessary for all biological processes, chloroplasts influence all aspects of the plant's life. The communication and coordination of gene expression between chloroplasts and nucleus is reciprocal and under a tight control. The knowledge of the processes in plants, especially those influenced by environmental changes, is important for agriculture, medicine and potentially also for renewable energy source production.

For specific determination of which components are involved in gene expression in chloroplasts and the manner of their action, *in organello* assays are the most practical tools for research. Experiments with isolated intact chloroplasts give the advantage of controlled environment where chloroplasts are exposed only to substances of choice. *In organello* assays were for example used to identify targeting of mustard nuclear-encoded transcription factor cpCK2 α to chloroplasts and its association with plastid RNA polymerase (Ogrzewalla et al., 2002), verification that excessive large subunits of ribulose biphosphate carboxylase are degraded in chloroplasts (Roy et al., 1982) and discovery of specific import into thylakoid lumen and intraorganellar routing of nuclear-encoded subunits of PSII in correlation with physiological requirements (Clausmeyer et al., 1993). Unfortunately the usual rapid decline in function and intactness of chloroplasts from higher plants after isolation has been limiting the range of application of *in organello* assays (Nivison and Jagendorf, 1984).

The robustness and *in vitro* longevity of chloroplasts from siphonaceous algae that form endosymbiosis with sacoglossan molluscs have been reported and investigated in more detail previously (Trench and Ohlhorst, 1976; Grant and Borowitzka, 1984). The remarkable stability of these chloroplasts was mainly in focus due to the acquisition of photosynthesis by the animal host. The activity of isolated chloroplasts from *V. litorea* has also been compared to other higher plants chloroplasts before (Green et al., 2005) but never in context of their better suitability for *in vitro* experiments than chloroplasts from well studied and readily accessible plant species. The objective of this project was to establish a protocol for culturing of *Vaucheria litorea* and for isolating intact chloroplasts from this alga. Further transcriptional and translational experiments should verify the structural and functional stability of these chloroplasts in comparison to chloroplasts from a higher plant model organism, *Pisum sativum*. The main goal was to investigate the potential of plastids from *V. litorea* for *in organello* systems involving transformation and protein expression in chloroplasts.

To be able to express the introduced genes, the isolated chloroplasts must sustain both intactness and transcriptional and translational activity over an extended period of time after isolation. The chloroplasts must be also robust enough to endure the insertion of foreign DNA either by chemical transformation or by electroporation (Daniell and McFadden, 1987; To et al., 1996). In this project, the ability of the isolated chloroplasts to retain the structural intactness was followed by phase contrast microscopy. Run-on transcription assays were used to detect changes in overall transcriptional activities. To investigate if there were some specific variations in transcription, radioactively labeled transcripts were hybridized to probes targeting mRNAs of major organellar photosynthetic genes and genes necessary for plastid protein translation. Finally, the persistence of translation in isolated chloroplast was estimated with hybridization assays following the incorporation of ³⁵S-methionine into *de novo* synthesized proteins over 24 hour period. The design of the construct that will be used for further transformation experiments, and the cloning steps done so far, are also described in this thesis.

2. Materials and Methods

2.1 Materials

2.1.1 Vaucheria and pea – media for cultivation and chloroplast isolation

2.1.1.1 Culture media for *Vaucheria bursata*

The initial culture of *V. bursata* was obtained from the lab of Prof. P. Kroth (Konstanz, Germany). Being a freshwater alga common in fast-flowing rivers or streams rich on nutrients (John et al., 2002) it requires an enriched medium for its growth *in vitro*. The medium described by Linne von Berg and Kowallik (1988) in a study on the plastid genome of this alga was used also in this project.

BM medium for *Vaucheria bursata*:

965ml dH₂O

5ml Ca(NO₃)₂ x 4H₂O (0,8g/100ml)

5ml K₂HPO₄ x 3H₂O (0,26g/100ml)

5ml MgSO₄ x 7H₂O (0,5g/100ml)

5ml Na₂CO₃ (0,5g/100ml)

5ml FeIII-Citrate x 5H₂O (0,125g/100ml)

0,9ml Citric Acid (5g/100ml)

5ml soil extract

5ml micronutrient solution

The pH was adjusted to 7,2 with Citric Acid and the medium was autoclaved. After cooling down, 1ml of sterile freshly filtered BM vitamins solution was added per liter of BM medium.

BM vitamin solution:

1mg Biotin

500mg Vitamin B₁₂

20mg Thiamin-HCl

Table 1: Media for micronutrient solution

Solution I and II were prepared and autoclaved separately and combined when cooled down.

Chemicals and concentrations		Solution I	Solution II
ZnSO ₄ x 7H ₂ O	(0,1g/100ml)	1ml	---
MnSO ₄	(0,1g/100ml)	2ml	---
H ₃ BO ₄	(0,2g/100ml)	5ml	---
Co(NO ₃) ₂ x 6H ₂ O	(0,02g/100ml)	5ml	---
Na ₂ MoO ₄ x 2H ₂ O	(0,02g/100ml)	5ml	---
CuSO ₄ x 5H ₂ O	(0,5mg/100ml)	1ml	---
H ₂ O		881ml	100ml
FeSO ₄ x /H ₂ O		---	0,7g
EDTA		0,4g	0,4g

2.1.1.2 F/2 medium for *Vaucheria litorea*

The initial culture of *V. litorea* was obtained from Prof. M. Rumpho (Orono, Maine, USA). The alga was further grown in a modified f/2 medium and enriched quarter-strength sea water as described by Green et al. 2005:

400µl Trace metal solution

400µl NaH₂PO₄ (0,5g/100ml)

400µl NaNO₃ (7,5g/100ml)

Nutrients were combined and filled up to 1l with ¼ strength artificial sea water (ASW) and autoclaved. When cooled down, medium was added 400µl of sterile freshly filtered BM vitamin solution (Acrodisc Syringe filter, 25mm 0,2µl Supor membrane).

Trace metal solution (solutions added to 150ml dH₂O, final volume adjusted to 250ml, autoclaved and cooled down before use):

250µl CoCl₂ x 6H₂O (1g/100ml)

250µl MnCl₂ x 4H₂O (1,8g/100ml)

250µl CuSO₄ x 5H₂O (98mg/100ml)

250µl Na₂MoO₄ x 2H₂O (630mg/100ml)

250µl ZnSO₄ x 7H₂O (2,2g/100ml)

1,25g EDTA, ferric Na-salt

¼ ASW:

- 8,25g sea salt (Instant Ocean, Aquarium Systems, France) dissolved in 1l dH₂O

2.1.1.3 Media for chloroplast isolation – Vaucheria

Homogenization buffer:

330mM Sorbitol (12,024g/200ml)

50mM Hepes (10ml 1M stock solution/200ml)

1mM MgCl₂ (0,2ml 1M stock solution/200ml)

1mM EDTA (0,4ml 0,5M stock solution/200ml)

0,2% BSA (0,4g/200ml; added fresh before homogenization)

Washing buffer:

- same as homogenization buffer excluding BSA

Table 2: Solutions for Percoll step-gradient

Chemicals	30% Percoll (10ml)	80% Percoll (5ml)
330mM Sorbitol	0,6g	0,3g
50mM Hepes (pH 7,6)	0,5ml 1M	0,25ml 1M
Percoll	3ml	4ml

2.1.1.4 Media for chloroplast isolation – pea

Homogenization buffer:

330mM Sorbitol (60,12g/1L)

20mM MOPS (4,19/1L)

13mM Tris (1,57g/1L)

3mM MgCl₂ (3ml 1M/1L)

0,1% BSA (1g/1L; added fresh before homogenization)

Washing buffer:

330mM Sorbitol (60,12g/1L)

50mM Hepes/KOH pH 7,6 (50ml 1M/1L)

3mM MgCl₂ (3ml 1M/1L)

Percoll step gradient

- same as for Vaucheria

2.1.2 Solutions for run-on analysis and dot blot hybridization

50µl 2x mix:

0,4 µl RNase-inhibitor (40u/µl)

5 µl 1M HEPES/KOH pH8

1 µl 1M MgCl₂

10 µl 250 mM K-Acetate

1 µl 1M DTT

10 µl 1,25 mM of each nucleotide, except of the one that is radioactively labeled

1 µl Heparin (50mg/ml)

1,6 µl dH₂O

1ml Stop-buffer:

50 µl 1M Tris pH8

50 µl 0,5M EDTA

0,05g Na-Sarcosinate

Prehybridization solution (same as hybridization solution without the sample)

5x SSC

5x Denhardt's solution (2% BSA, 2% Ficoll, 2% Polyvinylpyrrolidon PVP-360)

0,5 % SDS

40µg/ml denatured Herring sperma DNA (on 90°C first)

2.1.3 Solutions for *in organello* translation assay

2X SDS-PAGE Sample buffer:

125 mM Tris-HCl, pH 6,8

20% (v/v) Glycerol

4% (w/v) SDS

200mM DTT

0.1% (w/v) bromphenol blue

10x Laemmli running buffer:

250mM Tris

1,92M Glycerol

1% SDS

Coomassie staining solution:

0.1% coomassie R-250

40% methanol

10% acetic acid

Destaining solution:

7.5% acetic acid

10% methanol

2.1.4 Primers, bacterial strains and vectors

Table 3: Primers, restriction enzyme specificity and cut sites (in bold) and expected fragment sizes of the amplified PCR products:

Primer	Sequence	Enzyme specificity	Expected size
<i>ppsba</i> -pea fwd	5'- cccaagctt tactccatccgactag-3'	HindIII,	424bp
<i>ppsba</i> -pea rev	5'- cgggatcc gtttcgctatcgcgtc-3'	BamHI	
<i>ppsba</i> -V.l. fwd	5'- ccccaagctt ttacctattattgttgcgcgtc-3'	HindIII	1119bp
<i>ppsba</i> -V.l. rev	5'- cgggatcc ccataagctaagtcttcacgtc-3'	BamHI	
<i>egfp</i> fwd	5'- gggatc cttaatggtgagcaagggc-3'	BamHI	741bp
<i>egfp</i> rev	5'- gctctagat cattactgtacagctc-3'	XbaI	
T1 fwd	5'-aaggaaaa agcgccgctt caccgacaaacaagag-3'	NotI	120bp
T1 rev	5'-gc gagctc ggaaaacatccatgctag-3'	SacI	

Table 4: vectors and bacterial strains

Vector/strain	specificity
pB7FWG2	eGFP
C105220, U15884, U15978, U67823	<i>rnnB</i> T1 terminator
pGEM-T	Cloning vector, ampicillin resistance
pBS SK+	Cloning vector, ampicillin resistance
<i>E. coli</i> DH5 α	Bacterial strain with reduced endogenous nuclease activity, used for plasmid multiplication

2.1.5 General media

DNA extraction buffer:

2% CTAB (N-Cetyl-N,N,N-trimethylammoniumbromid)

100mM Tris/HCl, pH 8,0

1,4M NaCl

20mM EDTA

1% agarose gel:

0,5g agarose

50ml TAE buffer

0,5 μ l 1% ethidium bromide

TAE buffer

40mM Tris acetate

1mM EDTA

pH 8,5

10x bromophenol blue loading dye

50% glycerol

0,1% bromphenol blue

After combining of all ingredients the pH of the dye solution was adjusted with a few drops of NaOH until the color of the dye got blue. Bromphenol blue migrates through the gel in similar manner as DNA and is used as an indicator of the progress of separation.

LB medium:

Bacto-Tryptone 10g

Bacto-Yeast extract 5g

NaCl 10g

dH₂O to 1L, pH adjusted to 7,0

TBE buffer

89mM Tris base

89mM Borate

2mM EDTA

2.2 Methods

2.2.1 Cultivation of *Vaucheria bursata* and *Vaucheria litorea*

All bottles used for algal cultures were new and never washed with a detergent, only autoclaved before use. All handlings with the alga were done on a sterile bench and all equipment used was sterilized with 70% EtOH and flamed. Few threads of algae were inoculated in 20ml of medium in a small bottle with a lid. When the algae had grown sufficiently it was transferred into a large 2,5l Erlenmeyer flask with a cotton prop and sealed with aluminum foil. Both *V. bursata* and *V. litorea* were grown for minimum 6 weeks before use at 20°C with 16/8 hours on/off light regime. The cultures were aerated by manual swirling once a day.

2.2.2 Isolation of intact chloroplasts

Intact chloroplasts can be isolated from algae and higher plants by using homogenization and differential centrifugation. After only one centrifugation, the crude chloroplast pellet is a combination of intact and broken plastids and still contains some plant debris. The chloroplasts can be further purified by isopycnic centrifugation on discontinuous Percoll gradient. During the isolation, it is important to adjust the osmotic value of all media to that of the cell to avoid osmotic shock and chloroplast burst. As a compound with low osmolarity

consisting of silica beads of various sizes, Percoll is very suitable for separation of chloroplasts on a density gradient. It also helps to remove harmful phenolic compounds that together with released proteases and lipases can destroy the isolated chloroplasts (Seigneurin-Berny et al., 2008).

When observed with phase contrast microscopy, structurally intact chloroplasts appear bright with a light circle around referred to as a halo. Broken chloroplasts are dark, slightly larger, granulated and without halo (Walker, 1965). The structural intactness of isolated chloroplasts from *V. litorea* and *P. sativum* was examined with phase contrast microscopy using Leica Leitz DM RBE microscope and 400x magnification. The ratio of viable against broken chloroplasts was determined with Thoma counting chamber.

2.2.2.1 Isolation of intact chloroplast from Vaucheria

All steps were conducted at 6°C using pre-cooled media and equipment. The algae were collected with tweezers, washed twice in fresh growth medium and weighed. The filaments were cut with a razor blade and then grinded gently in a few milliliters of homogenization buffer. The volume of the homogenate was adjusted to 80ml (for wet weight of the algae 1-4g) and filtered through two layers of Miracloth moistured in advance with the homogenization buffer. The filtrate was filtered again through a set of micro sieves (45µm large pores placed on top of a 38µm). The filtered homogenate was centrifuged in polypropylene 40ml centrifuge tubes at 4°C and 1.930 g for 5' with the brake off (Beckmann JA 25.50 fixed angle rotor). The pellet was resuspended gently with a brush in a small volume (for 1-4 g of algae 100 – 200µl) of homogenization buffer and 150µl of the chloroplast suspension were loaded onto a Percoll step-gradient (2ml tubes, 500µl 80% Percoll on top of 1000µl 30% Percoll) and centrifuged at 4°C and 8.000 g for 20' with the brake on. Intact chloroplasts were collected at the 80%/30% interface with a cut pipette tip, washed in 3x volume of the pellet with washing buffer and centrifuged at 4°C, 3.500 rpm for one minute with slow deceleration. The washing step was repeated but only with 2x washing buffer volume. The final chloroplast pellet was resuspended in a small amount of washing buffer (100µl or less) and the tube was covered with aluminum foil and placed on ice.

2.2.2.2 Isolation of intact chloroplasts from pea

Thirty grams of 10 days old plant material (leaves and stems) were harvested with scissors and homogenized with 150ml of homogenization buffer in a Waring blender by 3 short (less than a second) bursts. The homogenate was filtered through two layers of Miracloth moistured in advance with the homogenization buffer. The filtered homogenate was centrifuged in 4 polypropylene 40ml centrifuge tubes at 4°C and 1.930 g for 5' with the brake off (Beckmann JA 25.50 fixed angle rotor). Each pellet was resuspended in 1ml of homogenization buffer and 1ml was loaded on Percoll step-gradient (15ml Falcon tubes, 5ml 30% Percoll on top of 2,5ml 80% percoll) and centrifuged for 20 min. at 6 000 rcf and 6°C in a swing out rotor (Heraeus Megafuge 1.0) with the break off. Intact chloroplasts were collected and washed as in the isolation protocol for *Vaucheria* and the final pellet was resuspended in 500µl of washing buffer.

2.2.3 Run-on transcription analysis

Run-on transcription assay is a technique that measures the rate in transcriptional activity as a variable of time or of particular cellular/organellar conditions (Karam et al., 1991). In chloroplast run-on transcription reaction using heparin, the labeled nucleotides are incorporated into elongating RNAs of the genes which transcription has already been initiated at that particular time. They are incorporated by RNA polymerases that are already attached to the DNA. Heparin is known to limit transcription initiation, but does not affect the elongation (Deng et al., 1987). This method was applied to measure the overall transcriptional activity in isolated chloroplasts at different time points after isolation.

The concentration of isolated chloroplasts was estimated using Thoma counting chamber and adjusted to 1×10^6 CP/µl. The tube with the chloroplast suspension was covered with aluminum foil and kept on ice. 1µl of the chloroplast suspension was added into a reaction mix (2,5µl 2x mix, 1µl dH₂O, 0,5µl ³²P-CTP (10µCi/µl; PerkinElmer)) and pre-heated to 30°C. The run-on transcription reaction was started immediately by pipetting the solution several times in order to disrupt the chloroplast membrane (Deng et al., 1987). Samples were incubated at 30°C for 6 minutes. The reaction was stopped with 1µl of stop buffer and samples were stored at -20°C until the measurement of incorporated radioactive nucleotides. Two independent reactions were incubated at each time point.

For determination of the relative incorporation the volume of each reaction was adjusted to 14µl with dH₂O and 2 x 1µl and 2 x 5µl from each sample was applied on a Whatman DE81

as described (Krause et al., 1998). Nucleotides not incorporated into the RNA were removed by washing the filters with 5µl of applied sample 5 x 4 minutes with 0,5M Na₂HPO₄, 2x 1 minute with dH₂O and 1x 1 minute with 100% EtOH. Filters with 1µl of the sample applied were used as references for the total amount of radioactivity used in each reaction. Radioactivity was measured with liquid scintillation analyzer (TriCarb 2300TR, PerkinElmer) in 2ml of Ultima Gold scintillation liquid (PerkinElmer).

2.2.4 Dot blot hybridization analysis

Nucleic hybridization methods are based on the complementary reversible binding of two strands of nucleic acids depending on the sequence homology. They can be used both for detection and relative estimation of the concentration of either DNA or RNA molecule of interest but requires at least partial sequence knowledge of the targeted molecule. There are various applications (virus detection, microbial community analysis and others) and different methods developed for signal detection (radioactively labeled probe or target molecule, use of secondary probe with enzymatic activities and others) (Evans and Kaslow, 1997).

In this project the radioactively labeled cpRNA was hybridized to immobilized probes designed to target mRNA for selected genes (table 5). The whole chloroplast RNA was labeled at different time points after isolation and dot blot hybridization was used to detect changes in transcriptional activity. Probes were also applied in concentration gradient to detect possible decrease in concentrations of target mRNAs (Deng et al., 1987).

Table 5: Specificity of short (40 – 45bp) ssDNA oligonucleotide probes used for dot blot hybridization assay, probes targeting chloroplast mRNA designed separately for *Vaucheria* and pea

Gene	Specificity
<i>psaA</i>	Chlorophyll apoprotein of Photosystem I
<i>psbA</i>	Quinone-binding protein of Photosystem II
<i>psbD</i>	Chlorophyll apoprotein of Photosystem II
<i>rbcL</i>	Large subunit of ribulose bisphosphate carboxylase
<i>rrn16</i>	16S rRNA
<i>rrn23</i>	23S rRNA
<i>trnE</i>	Glutamine tRNA
pBS SK+	MCS in cloning vector pBS SK+, negative control of binding specificity

Each oligonucleotide probe (100 μ M stock solution) was diluted to 1000fmol/ μ l and aliquoted into 3 dilutions (1 – 32x10³ fmol/100 μ l, 2 – 8x10³ fmol/100 μ l, 3 – 2x10³ fmol/100 μ l). Hundred microliters of 0,8M Na OH/0,02M EDTA was added into 100 μ l with each dilution; probes were heated to 100°C for 10 minutes and chilled on ice. 200 μ l of each probe dilution was applied to the pre-moistured membrane nitrocellulose underlaid with a Whatman paper using a dot blot apparatus. The wells of the apparatus were rinsed with 500 μ l of dH₂O prior and 500 μ l of 0,4M NaOH after the application of the probes. Oligos were cross linked to the membrane with UV Stratalinker and visualized by staining of the membrane for 15 minutes in 0,5M Na-acetate/0,04% methylene blue solution.

At each time point the chloroplast RNA was labeled with radioactive nucleotides (³³P-UTP; 10 μ Ci/ μ l, PerkinElmer) using a large run on transcription assay (50 μ l 2x mix, 20 μ l dH₂O, 10 μ l P-UTP, 20 μ l of chloroplast solution and 20 μ l stop buffer). The concentrations of the chloroplast solutions were 1x10⁶/ μ l in case of pea, 0,4x10⁶/ μ l in case of *V. litorea* and 0,5x10⁶ in case of *V. bursata*. Non-incorporated nucleotides were removed from the assay after the reaction with MicroSpin^{TMG} kit for purification of labeled oligonucleotides. Prior to hybridization membranes were incubated in pre-hybridization solution for 1 – 3 hours at 60°C. Hybridization was carried out in 120ml pre-heated hybridization solution at 62°C for 16 – 18 hours, rotating in the hybridization furnace (HYBAID Mini 10).

Membranes were washed 4x in 75ml of SSC/0,1% SDS solution (1 – 2x SSC, room temperature, short hand shake; 2 – 2x SSC, 62°C, 10 minutes rotating in hybridization furnace; 3 – 1x SSC, 42°C, 10 minutes shaking; 4 – 0,5x SSC, 42°C, 10 minutes shaking) and sealed immediately after washing in thin plastic foil. The membranes were exposed to phosphoscreen-K in dark for 5 weeks and radioactivity was detected by Personal Imager FX (BioRad).

2.2.5 *In organello* translational assay

To study translational activities in isolated organelles (both chloroplasts and mitochondria), proteins can be radioactively labeled with methionine and cysteine containing ³⁵S. In translational reaction amino acids are transported into the organelles and incorporated in the synthesized proteins. This method can be used for detection of overall change in translational activities and for localization of proteins in subcellular compartments (Leister and Herrmann, 2007; Green et al.2005).

2.2.5.1 Protein labeling

V. litorea and *P. sativum* chloroplasts were isolated according to their specific protocol. In each reaction 95µl of chloroplast/washing buffer (WB) suspension was added ATP, all essential amino acids (except of methionine), dithiothreitol (DTT), ³⁵S-methionine/cysteine (10µCi/µl, PerkinElmer) and 2x WM with final concentrations 10mM ATP, 40µM each amino acid except methionine, 10mM DTT, 50µCi ³⁵S-met/cys and 1x WB as described (Barkan et al., 1994). Samples were incubated at 28°C for 20 min. The labeling reaction was stopped by the addition of 7µl of 200mM methionine and 150µl of ice cold WB. After mixing the solution gently, 25µl were taken out for detection of incorporated radioactivity as described in chapter 2.2.3. The rest was centrifuged for 15 seconds at 13 000 rpm. The chloroplast pellet was frozen down immediately in liquid N₂.

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

SDS-PAGE is a method used for separating proteins according to their size. Sodium dodecyl sulfate (SDS) is an anionic detergent that binds to most proteins in constant weight ratio. Proteins heated in presence of SDS denature into their primary polypeptides and gain an overall identical negative charge density. These polypeptides, migrating in an electric field towards the positive anode, can be then separated in a porous gel according to their size with smaller proteins migrating faster than the larger ones (Hames, 1998). In this project the total chloroplast protein labeled at certain time points after isolation with ³⁵S-met/cys was separated on gel in order to be able to visualize the change in translation rate over time.

Table 6: Solutions and concentrations for the SDS-PAGE

Solutions	12 % Resolving gel (10ml)	4% Stacking gel (5ml)
30% Acrylamid	4 ml	0,75 ml
H ₂ O	2,2 ml	3 ml
1M Tris	3,8 ml (pH 8,8)	1,3 ml (pH 6,8)
10% SDS	100µl	50µl
10% APS	50µl	50µl
TEMED	5µl	8µl

The equipment used for casting the gel was washed with 70% EtOH to remove fat that would restrict polymerization. The solution for resolving gel was applied first up to 1cm beneath the

combs and sealed from surrounding air with 80% isopropanol until the gel solidified. The isopropanol was removed; space washed with dH₂O and the stacking gel was cast on top of the resolving gel with combs mounted on top.

Thawed labeled chloroplast pellet was resuspended by vortexing with equal volume of 2x SDS-PAGE Sample buffer (30µl), heated for 10 min. at 95°C and chilled briefly on ice. Large insoluble particles in the samples as debris were pelleted by centrifugation for few seconds and the supernatant protein sample (50µl) was loaded on the stacking gel. The low pH in the stacking gel and the comparatively high concentration of Acrylamid in resolving gel concentrates all proteins at a narrow line at the beginning of the resolving gel after the voltage is applied. The gel was run for 2 h at 80V (BioRad PowerPac™). To visualize the separation efficiency the gel was stained for 1 h with coomassie, swirling. Before exposing the gel to a phosphoscreen-K, the gel was destained over night and dried with vacuum drier (Stab GEL SE1160) at 70°C. The change in radioactive labeling was detected with Personal Imager FX.

2.2.6 Cloning

2.2.6.1 DNA isolation

Both genomic and organellar DNA can be readily isolated from plant tissues of many species using the standard phenol-chlorophorm method. The DNA is made accessible by mechanical disruption of the rigid cell wall. At this step, the sample must be kept cold to inhibit the cellular degrading enzymes. Incubation of the sample in CTAB buffer further facilitates lysis of the cells. The DNA dissolved in water is separated from proteins, membranes and other insoluble metabolites with organic solvents chlorophorm and phenol. The DNA is then precipitated from the water solution with EtOH in presence of Na⁺ ions (Henry, 2001; Ausubel et al., 1988).

2.2.6.1.1 Isolation of total DNA from plant leaves of *P. sativum*

Eight to ten leaves of 6 week old pea plants (20°C, 16/8 on/off light regime) were frozen down with liquid N₂ and then grinded with pre-cooled pestle in mortar placed on ice. Two hundred milligrams of the leaf powder in an Eppendorf tube was added 600µl of Extraction buffer and vortexed for 30 seconds. Sample was incubated at 60°C for 30 min., added 600µl Phenol/Chloroform/Isoamyl alcohol (25:24:1), vortexed for 30 sec. and centrifuged for 10 minutes at 10 000 rpm. The upper aqueous phase was transferred to a new tube and filled up to 600µl with dH₂O. The centrifugation step was repeated together with 600µl Chloroform/Isoamyl alcohol (24:1). The upper aqueous phase (500µl) was gently mixed with

0,7 volumes (350µl) of Isopropanol and centrifuged for 20 min. at 14 000 rpm and 4°C. The DNA pellet was washed with 1ml ice-cold 70% Ethanol and centrifuged 5 min. at 15 000 rpm and 4°C. The supernatant was removed completely and the pellet dried at room temperature for 15 min. The dried DNA pellet was resuspended in 20µl of dH₂O. Concentration of total leaf DNA from *P. sativum* in 4 samples prepared according to this protocol was measured by Nano Drop 1000.

2.2.6.1.2 Isolation of plastid DNA from chloroplasts of *V. litorea*

Chloroplasts were isolated according to the protocol for chloroplasts isolation. After the percoll gradient and microscopy both broken and intact chloroplast fractions were combined, centrifuged down for 4 min. at 14 000 rpm and the pellet was frozen down in liquid N₂. The next day the ice cold but thawed chloroplast pellet was resuspended with 600µl of DNA extraction buffer and DNA was isolated according to the protocol for DNA isolation from leaves. After the final centrifugation the DNA pellet was resuspended in 10µl of dH₂O and the concentration measured with Nano Drop 1000. The concentrated chloroplast DNA was diluted to 100ng/µl with dH₂O and kept at -20°C until further use.

2.2.6.2 PCR

Polymerase chain reaction (PCR) is a method that uses a thermostable DNA polymerase enzyme to amplify a DNA sequence of interest. It is necessary to know the base pair composition of the short regions flanking the sequence to be amplified in order to design the primers (short oligonucleotides complementary to one of the strands of the flanking regions in 5'=>3' direction) that initiate DNA synthesis. The reaction proceeds in three steps – heat denaturation of the hydrogen bonds in DNA double helix producing two single strands, annealing of the primers to each strand at the end of the sequence to be amplified and elongation step during which the polymerase enzyme attaches strand-complementary nucleotides to the 3' end of the primer and continues to synthesize the complementary strand. To obtain a satisfactory amount of DNA these three steps must be repeated in 20 or more cycles (Reece 2004, Wilson and Walker 2000).

PCR was used to amplify the promoter sequences for *psbA* gene (*p_{psbA}*) from chloroplast DNA of *Pisum sativum* (Khanna et al., 1991) and *Vaucheria litorea* (1000 bp upstream of *psbA* start, the chloroplast genome sequence available at public databases), the sequence coding for an enhanced green fluorescent protein (*egfp*) from plasmid pB7FWG2 (Gateway

cloning vector for *Agrobacterium*-mediated plant transformation, Karimi et al., 2002) and the terminator sequence *rrnB* T1 (*E. coli* ribosomal RNA operon T1 terminator, Nojima et al., 2005) from plasmids with pENTR, D-TOPO and pUNI51 backbones (C105220, U15884, U15978 and U67823 respectively). Primers were designed to contain specific restriction sites for further cloning of the sequences into the pBluescript SK+ vector used further for chloroplast transformation.

Table 7: Standard PCR reaction mix per 20µl of total volume

Reagents	Volume (in µl)
10x reaction buffer	2
dNTPs (2,5mM)	1,6
forward primer (5µM)	1
reverse primer (5µM)	1
Taq DNA polymerase (2u/µl)	0,2
DNA template (50ng/µl dil.)	1
dH ₂ O	13,2

Table 8: PCR program

Step	Time	Temperature
Initial denaturation	2 min	95°C
Denaturation	Repeated 33x	30 sec
Annealing		30 sec
Elongation		1 min - <i>egfp</i> , <i>ppsbA</i> 30 sec - <i>rrnB</i> T1 1,2 min - <i>ppsbA</i> V.l.
Finishing elongation	10 min	72°C
Cooling	∞	10°C

After the amplification, 1,2µl of 10x Bromphenol-containing loading buffer was added to the reaction mixtures containing the amplified fragments. Fragments were separated from the template DNA by agarose gel electrophoresis and either cut out of the gel and purified or purified directly from the PCR mixture after verification of the size for further subcloning into a helper vector pGEM-T.

2.2.6.3 Agarose gel electrophoresis

DNA or RNA molecules of different sizes can be separated on an agarose gel due to the negative charge of the phosphate groups in the backbone of the nucleic acids. Agarose is a gelatinous polysaccharide that forms a porous matrix after dissolving in hot water and cooling down. Embedded in the agarose gel, nucleic acids move to the positive pole of the electric field through the gel with shorter molecules moving faster than the longer molecules. The resolution capacity of this method can be adjusted by the concentration of agarose in the gel with higher amount of agarose being suitable for separation of shorter fragments and opposite, lower concentration for larger fragments. DNA can be visualized on the gel with Ethidium Bromide that intercalates in the double helix and fluoresces under UV-light. Agarose gel electrophoresis can be simultaneously used for determination of the DNA concentration in the specific fragment by comparison of the intensity of the band to the standard molecular weight marker of a known concentration.

Agarose gel electrophoresis was primarily used to verify the expected size of the amplified DNA sequences, but also to separate the fragments from the template DNA, unincorporated nucleotides, polymerase and buffer salts. For size estimation, 10 μ l of molecular weight marker (GeneRulerTM 1Kb DNA ladder, Fermentas) were loaded in the first well. The gels were run at 100V for approx. 60 min. The fragments were visualized by UV light using a GelDoc 2000 (BioRad).

2.2.6.4 Purification of the DNA fragments from the gel

Fragments amplified by PCR can be purified directly from the PCR reaction or from the gel after the agarose gel electrophoresis. In this project The HiYieldTMGel/PCR DNA Fragments Extraction [Mini] Kit (RBC – Bioscience) was used for purification of the fragments from the gel. In this method the agarose gel is dissolved and the polymerase enzyme is denatured with guanidine thiocyanate, a chaotropic salt that interferes with hydrogen-bonds and other stabilizing non-covalent forces. In this solution DNA fragments bind to the glass fiber matrix of the spin column but other substances pass through. After a washing step, the DNA fragments between 50bp – 10Kb can be detached from the matrix and eluted with low salt buffer or water (www.biocompare.com/ProductDetails).

The amplified fragments of correct size were cut out of the gel with a scalpel while illuminated with UV-light and purified according to the enclosed manual. The DNA was eluted from the columns with 20µl of dH₂O and DNA concentration was measured with NanoDrop. This DNA was further used for ligation into the helper vector pGEM-T.

2.2.6.5 Subcloning of the DNA fragments into the pGEM-T-Easy vector

The pGEM[®]-T Vector is specifically designed for cloning of PCR products. It is pre-cut with EcoR V in the middle of a multiple cloning site (MCS) and added 3' terminal thymidine at both ends preventing recircularization. These overhangs are also compatible with a single deoxyadenosine overhangs created independently on the template by certain thermostable DNA polymerases at the ends of the PCR products. The pGEM-T is a high copy plasmid and can be purified in high numbers after transformation into suitable competent cells. The MCS is flanked with M13 and T7 and SP6 primer binding sites suitable for sequence verification of the inserted fragment (pGEM[®]-T and pGEM[®]-T Easy Vector Systems, Promega, Technical Manual No. 042, revised 11/98).

The amplified sequences were subcloned into the helper vector pGEM-T in order to create a large number of identical clones from a single PCR amplified sequence. The Taq polymerase used for PCR has not completely sufficient proof reading capacity creating a single nucleotide mismatch error once per 1000bp. Each pGEM-T vector can contain only one insert and under further transformation of the vectors into competent cells, each cell will contain only one pGEM-T plasmid. The DNA polymerase of competent *E. coli* has a much stringent proof reading and the plasmid is copied with no further errors during the bacterial growth. The correct sequence can be identified by sequencing when a single colony transformed with a pGEM-T containing the insert of interest is used for plasmid isolation.

The amount of reagents in the ligation reaction was reduced to half of the recommended standard reaction in the technical manual for pGEM-T vectors. The amount of PCR DNA was calculated according to recommended insert:vector molar ratio (3:1) using the reduced amounts for vector DNA:

$$\text{(ng of vector x kb size of insert x insert:vector molar ratio)} / \text{kb size of vector} = \text{ng of insert}$$

Ligation reaction (per 5µl total volume):

2X Rapid Ligation Buffer for T4 DNA Ligase 2,5µl

pGEM®-T Vector (25ng) 0,5µl

T4 DNA Ligase (3 Weiss units/µl) 0,5µl

deionized water to a final volume of 5µl

After combining all the reagents, the ligation reaction was incubated at room temperature for 1 hour and left at 4°C over night and if not immediately used for transformation, the reaction was stored at -20°C.

2.2.6.6 Preparation and transformation of chemically competent *E. coli* DH5α

The treatment of *E. coli* cells with Ca²⁺ and some other divalent or multivalent cations induces competence and makes these cells to take up DNA from the environment (Glover and Hames, 1995). With this method, the *E. coli* strain DH5α was transformed with the ligation reaction of the pGEM-T vector with the amplified fragments. Only one copy of the re-ligated plasmid can be stably introduced into each cell and further multiplied during the growth of the bacteria. These transformants can be selected for using ampicillin as a selection marker because of the presence of the ampicillin resistance gene in the plasmid.

A tip with few cells from single DH5α colony was transferred to a tube with 3ml of LB medium. The culture was incubated for 18h at 37°C shaking at 220 rpm and then transferred into 50ml of LB where the cells were further grown until the optical density of the culture reached OD(A₆₀₀) 0,35 – 0,4. After cooling on ice for 10 min. cells were centrifuged down at 5000 rpm at 4°C for 10 min. and the pellet was gently resuspended in 20ml ice cold 100mM CaCl₂. The suspension was left on ice for 10 min. and centrifuged as described above. The pellet was gently resuspended in 4ml 100mM CaCl₂ and left on ice for 30 min. After addition of 1ml 80% glycerol, aliquots of 50µl were transferred into 1,5ml Eppendorf tubes with a cut pipette tip and frozen immediately in liquid N₂. The cells were kept at -80°C until use.

Before transformation, cells were thawed on ice and used immediately after thawing. 2 – 5µl of the ligation reaction were added into 50µl of the competent cells, mixed gently by ticking against the tube and left on ice for 15 min. The mixing step was repeated and the reaction left on ice for additional 15 min. The cells were exposed to a heat shock of 42°C for exactly 90 seconds and were incubated at 37°C and 220 rpm for 30 – 60 min after addition of 750µl of

pre-warmed (37°C) LB medium. Four times 200µl of the suspension was spread on 4 agar plates and incubated for 18 h at 37°C.

2.2.6.7 Colony PCR

Bacteria originating from a single colony can be used directly as template for PCR because the initial denaturing heat disrupts the cell walls and make DNA accessible (Fuchs and Podda, 2005).

Even though both ends of the open pGEM-T vector are extended with 3' terminal thymidines to avoid self re-ligation these ends are not dephosphorylated and can be re-ligated by T4 ligase. The presence of the pGEM-T plasmid containing the insert of interest in the colonies growing on ampicillin plates was verified by PCR using the cells directly as a DNA template. Colony PCR was also used to detect cloned fragments in the entry vector pBS SK+ after ligation reaction.

Each colony chosen for colony PCR was transferred to a new ampicillin plate with a pipette tip and remains of the cells from the tip were mixed into the PCR reaction. The PCR reaction mix was set up as described in table 7, except that the total volume was adjusted to 20µl without a DNA template. In each screen, the primers specific for the pGEM-T vector were used (M13 or T7 fwd and M13 or SP6 rev), sometimes in addition with one of the insert specific primers. The program for the PCR was the same as used before (table 8). The amplified products were separated from the template DNA with 1% agar gel containing TBE buffer and visualized as described in agarose gel electrophoresis protocol. Positive colonies showed a fragment of the size combining the insert flanking regions and the insert size.

2.2.6.8 Plasmid isolation

Plasmid DNA can be rapidly isolated from bacterial cultures using differential alkaline denaturation. In this method cells are lysed with combination of sodium dodecyl sulfate (SDS) and NaOH. Both chromosomal and plasmid DNA and proteins are denatured in this process but after the neutralization of the solution with Na-acetate only the plasmid DNA can re-anneal and stay dissolved in the solution. Chromosomal DNA and proteins stay in the complex with SDS and NaOH and are removed by centrifugation. Plasmid DNA can be then concentrated by precipitation with ethanol (Birnboim and Doley, 1979).

The HiYield™ Plasmid Mini Kit (RBC Bioscience) based on adjusted alkaline denaturation was used to purify the pGEM-T vectors from *E. coli* DH5 α that were identified as positive with the colony PCR.

2.2.6.9 DNA sequencing

The exact order of base pairs in a certain DNA sequence can be determined by dye-terminator sequencing. In this method the double stranded DNA fragment to be analyzed is mixed with a single primer binding to a strand complementary to the strand to be sequenced, DNA polymerase and nucleotide mixture containing ordinary dNTPs and ddNTPs labeled with a fluorescent dye but missing 3'-OH group at the polymerization site. In a reaction based on the same principle as PCR, insertion of ddNTP terminates the extension of that particulate strand. The insertion of these nucleotides is random but after a series of cycles the sequencing reaction contains mixture of copies of one strand ended at each base. There are four different dye-labels specific for each nucleotide. During the separation of the strands by size in a capillary electrophoresis the dyes of strands of equal length are excited passing a laser and the specific light emission is detected (Reece 2004).

DNA sequencing was used to verify the exact sequence of the fragments cloned into the pGEM-T helper-vector and pBluescript SK+ entry vector and was carried out using BigDye™ Terminator v3.1 Cycle Sequencing Reaction Kit.

Table 9: Standard sequencing reaction mix:

Reagents	Volume (in μ l)
5x BigDye Sequencing buffer	2
BigDye 3.1	0,5
M13 primer forward/reverse (3.2 μ M)	0,5
DNA template	25 – 50ng
dH ₂ O	Up to 10 μ l

Table 10: Sequencing reaction:

Step		Time	Temperature
Initial denaturation		1 min	96°C
Denaturation	Repeated 24x	10 sec	96°C
Annealing		5 sec	50°C
Elongation		4 min	60°C
Cooling		∞	10°C

The inserts were sequenced in both directions using M13 primers that flank the insertion site of pGEM-T vector and extending the sequence with the insert with additional 116bp at the 5' end and 120bp at the 3' end. After the reaction was completed the volume was adjusted to 20µl. The samples were sequenced at MBI UiTø and the results analyzed with BioEdit.

2.2.6.10 Restriction digestion

Restriction endonucleases are bacterial enzymes that cleave dsDNA at specific sites by recognizing specific short sequences and produce restriction fragments. These enzymes can be used for “excision” of a DNA sequence of interest from plasmids or chromosomal DNA if there are sites that are recognized by specific restriction endonucleases flanking the DNA region. After the reaction, the DNA restriction fragment can be separated from the remaining molecule by agarose gel electrophoresis and purified from the gel. Enzymes that cleave each strand of the dsDNA at shifted positions within the recognition site create few base pairs long single stranded overhangs at the ends of the fragments. These “sticky” overhangs bind complementary to other DNA molecules digested with the same enzyme (Wilson and Walker, 2000).

This method was used to recover the *ppsba* for pea and *V. litorea*, *egfp* and *rnnB* T1 fragments from the specific helper pGEM-T vectors after confirmation of the correct sequences and multiplication of plasmid DNA by *E. coli* DH5a. The specificity of the restriction enzyme was determined by the sites within the primer sequences used for the original PCR amplification of the fragments (table 3) and now flanking the inserts in pGEM-T. These specific enzymes were also used to cleave the entry vector pBS SK+ to create complementary sticky ends for further cloning of the fragments.

Table 11: Restriction digestions of pGEM-T helper vectors with inserts and pBluescript SK+

Fragment/plasmid	DNA	Enzymes	Buffers	conditions
pGEM- <i>rnnB</i> T1	3,7µg (120bp)	NotI 5u/SacI 10u (BioLabs) Double digest.	2x Tango TM Yellow (Fermentas)	20µl tot. V, 37°C overnight
pGEM-pea <i>ppsba</i>	2µg (424bp)	BamHI 4u/HindIII 8u (BioLabs) Double digest.	2x Tango TM Yellow (Fermentas)	20µl tot. V, 37°C for 2h
pGEM- <i>ppsba</i> V.1.	2µg (1119bp)	BamHI 4u/HindIII 8u (BioLabs) Double digest.	2x Tango TM Yellow (Fermentas)	20µl tot. V, 37°C for 2h
pBS SK+	2µg	NotI 5u/SacI 10u (BioLabs) Double digest.	2x Tango TM Yellow (Fermentas)	20µl tot. V, 37°C for 2h

The restriction fragments were separated by agarose gel electrophoresis, cut out and purified as described above. The NotI and SacI enzymes in pBluescript SK+ digest were deactivated by incubation at 65°C for 20 min. and the DNA 5'ends were dephosphorylated prior to gel electrophoresis with 1u of Alkaline phosphatase (Promega) for 30 min. at 37°C to avoid self religation. After unsuccessful digestion attempts of the pGEM-T with *egfp* insert with BamHI and XbaI (BioLabs), the *egfp* fragment was amplified by PCR using M13 fwd and rev primers which extended the fragment with 236bp.

Table 12: PCR amplification of *egfp* from pGEM-T

Reagents	Volume per reaction (in µl)	Master mix for 4 reactions (5x)
10x buffer for the polymerase	3	15
dNTPs (2,5mM)	1,6	8
M13 forward primer (5µM)	1	5
M13 reverse primer (5µM)	1	5
Taq DNA polymerase (2u/µl)	0,2	1
DNA (50ng/µl)	0,4	2
dH ₂ O	23,8	119

The PCR program was same as used previously. After amplification all reactions were combined (120µl) and DNA precipitated with 12µl 3M Na-acetate and 330µl 100% EtOH for 1h at -80°C and centrifuged for 5 min. at 14 000 rpm. DNA pellet was washed with 70% EtOH, dried and resuspended in 10µl of dH₂O. The extended *egfp* fragment and the pBluescript SK+ vector with *rnnB* T1 already inserted were digested with BamHI and XbaI in two steps with DNA precipitation step between the digestions.

Table 13: Two step restriction digestion of *egfp* fragment and of pBluescript SK+ with inserted *rnnB* T1

Reagents	<i>egfp</i> with BamHI (37µl tot. V)	<i>egfp</i> with XbaI (15µl tot. V)	pBS SK+- <i>rnnBT1</i> with BamHI (37µl tot. V)	pBS SK+- <i>rnnBT1</i> with XbaI (15µl tot. V)
Enzyme	10u	30u	10u	30u
Buffer	1x Tango™ Yellow (Fermentas)	1xNEB4 + 1xBSA (BioLabs)	1x Tango™ Yellow (Fermentas)	1xNEB4 + 1xBSA (BioLabs)
DNA	2,5µg	870ng	2,5µg	1,3µg
Conditions	3h at 37°C	37°C overnight	3h at 37°C	37°C overnight

After digestion, XbaI in pBluescript SK+ with *rnnB* T1 was deactivated at 65°C for 20 min. Plasmid ends were dephosphorylated with 1u of Alkaline phosphatase for 1 h at 37°C which there after got heat inactivated at 65°C for 20 min. The *egfp* fragment was cut out of gel after electrophoresis and purified.

2.2.6.11 Ligation

DNA ligases transfer an adenyl residue to the phosphate at the 5' terminus of one DNA molecule. It is then open to a nucleophilic attack by a hydroxyl group at the 3' terminus of a neighbouring DNA molecule in an immediate proximity. T4 DNA ligase originates from bacteriophage T4 and can be used for wide range of ligation reactions such as ligation of both cohesive and blunt DNA termini, oligoribonucleotide in RNA-DNA hybrids and others. It requires ATP and Mg²⁺ and is inhibited by high concentrations of Na⁺, K⁺, Cs⁺, Li⁺ and NH₄⁺. The advantage of this particular enzyme is that it has low efficiency in ligating

cohesive ends that contain mismatches and favors ligating complementary ends of restriction fragments digested with the same restriction endonuclease (Sambrook and Russell, 2001).

T4 DNA ligase was used for ligation of the *psbA* promoters, *egfp* and *rnnB* T1 into the entry vector pBluescript SK+ in the right sequence after the digestion with the corresponding restriction enzymes.

Table 14: Ligation reactions for *rnnB* T1 and *egfp* into pBluescript SK+

Reagents	<i>rnnB</i> T1	<i>egfp</i>
Vector DNA	148ng	65ng
Insert DNA	3ng	53ng
T4 DNA ligase (Fermentas)	1µl	2µl
Buffer (Fermentas)	2µl 10x T4 lig. Buffer	2µl 10x T4 lig. Buffer
dH ₂ O	3µl	12,5µl

The ligation reaction for *egfp* was calculated according to the recommended 3:1 insert/vector molar ratio as described in chapter 2.2.6.5. The recommendation was not followed in case of *rnnB* T1 due to the low concentration of the insert DNA. Both reactions were incubated for 1h at room temperature and at 4°C over night. Vectors with inserts were detected and purified by transformation into *E. coli* DH5α, colony PCR and plasmid isolation as described above.

3. Results

In this project both the structural and the functional stability of isolated chloroplasts from *Vaucheria litorea*, *Vaucheria bursata* and *Pisum sativum* was investigated. The structural intactness was estimated with phase-contrast microscopy. When observed with phase-contrast microscopy, the intact chloroplasts were highly refractive with a bright halo surrounding the organelle, the broken chloroplasts were dark with a weak halo or none at all (fig. 9). The relative amount of intact chloroplasts present in the solution at different time-points after isolation was determined by the ratio of the bright chloroplasts with halo against the total amount of chloroplasts (chapter 3.3).

The change in overall transcriptional activity of isolated chloroplasts was tested with run-on transcription assays using radioactively labeled nucleotides. Isolated chloroplasts were incubated with ^{33}P -UTP/ ^{32}P -CTP at different time-points after isolation and the RNA synthesis rate was detected by measuring the amount of incorporated radioactivity with a liquid scintillation counter (chapter 3.4).

The initial experiments revealed that the cultures of *V. litorea* and *V. bursata* were contaminated with bacteria. The motile bacteria were not removed completely during the chloroplast isolation and multiplied to large concentrations in the medium with isolated plastids when stored at 4°C (fig. 6). The experiments that were applied in attempts to obtain axenic algal cultures are outlined in chapter 3.1 and the modifications of the original protocol for chloroplast isolation from these cultures (Green et al., 2005) in chapter 3.2.

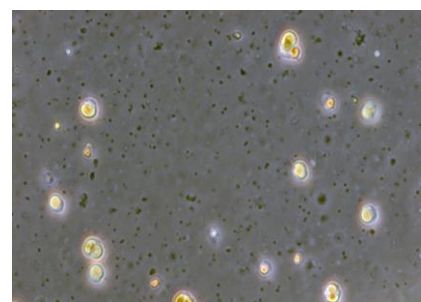


Figure 6: Isolated chloroplast of *V. bursata* , 2 days after isolation when stored at 4°C

Since the bacterial contamination could not be completely abolished, the specific incorporation of radioactive nucleotides into chloroplast RNA was checked by dot blot hybridization. The RNAs labeled in run-on experiment at time 0 and 4 were hybridized to blots with probes targeting genes coding for proteins that are involved in photosynthetic light reactions (*psbD*, *psbA* and *psaA*), large subunit of ribulose biphosphate carboxylase (*rbcL*),

ribosomal RNAs of small and large subunit (*rrn16* and *rrn23*, respectively) and transfer RNA specific for glutamine (*trnE*) (section 3.4).

In organello translation assays were used to verify that chloroplasts isolated from *V. litorea* and *P. sativum* were also translationally active for a period of time after isolation. Chloroplasts were incubated with ³⁵S-met/cys at 0, 4 and 24 hours after isolation and the total chloroplast protein from these plastids was separated by SDS-PAGE. The total amount of protein (both labeled with ³⁵S-met/cys and unlabeled) present in the isolated chloroplasts was visualized by staining with coomassie. The *de novo* synthesis of protein was detected as radioactive signal by Personal Imager FX after exposure of the gel to phosphoscreen-K (chapter 3.5).

The vectors that were designed to be used for transformation of isolated chloroplasts from *V. litorea* and *P. sativum* by electroporation and for expression of eGFP in these chloroplasts are shown in the appendix. The ability of the isolated chloroplasts to express eGFP would verify the suitability of these plastids for *in organello* translation experiments. The results from cloning experiments are summarized in chapter 3.6.

3.1 Efforts to obtain axenic Vaucheria cultures

Figure 7 demonstrates the set-up for removal of bacteria without use of chemicals that could damage the alga. The idea was to use the force of growing filaments through the agar towards the light while shedding off the bacteria that would not be able to move in the solid medium. The filaments grew successfully through both the soft (0,5%) and the more concentrated agar-medium (2%). The outer tips of the longest filaments were cut off with a sterile razor blade and inoculated in new sterile medium. The presence of bacteria was examined by spreading 200µl of the culture medium on LB agar plate and incubating in the growth chamber for *Vaucheria*. This method was not successful.

In further attempts to obtain axenic cultures it was used intensive rinsing of few of the filaments in 6 successive washes prior to inoculation into a new medium. This washing and inoculating step was repeated 6x with 1 h interval one day and 8x every ½ h the next day. Also the aplanospores were isolated from the cultures, rinsed in several successive washes and inoculated in 0,5ml of culture medium in reagent tubes. None of the few germinated aplanospores managed to grow further and form longer filaments.

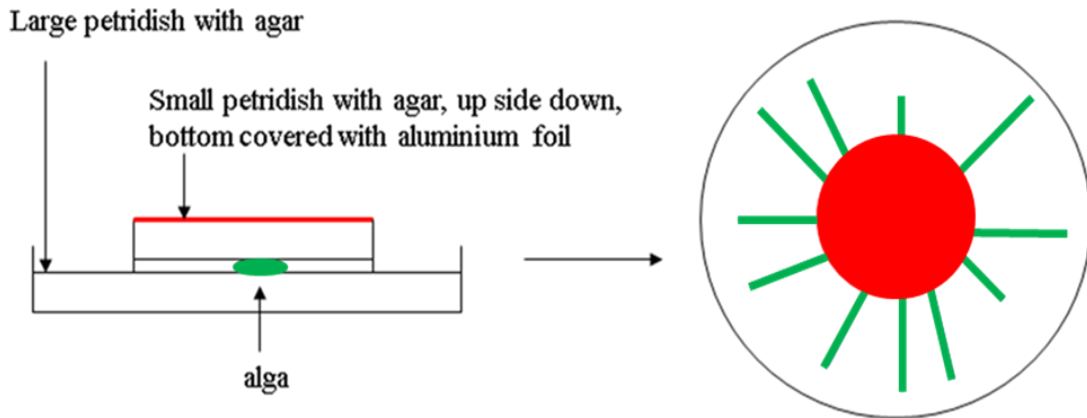


Figure 7: Agar set-up for removing the bacteria using phototropism. Few short filaments of the alga were placed in the middle of Petri dish containing medium solidified with 0,5% and 0,75% agar. The alga was then covered with Petri dish of smaller diameter filled with medium solidified with 2% agar turned upside down and with bottom covered with aluminum foil. The alga was growing through the agar in the large dish towards the light.

Finally, different antibiotics were used, both separately and in combination directly in the culture media but also in addition to the adjusted agar set-up. Ampicillin and carbenicillin, the antibiotics targeting bacterial cell wall synthesis and thus do not damage the chloroplasts, had no effect on the bacterial growth. Antibiotics impeding the bacterial RNA synthesis used in this project (chloramphenicol, tetracycline, rifampicin, kanamycin, gentamycin and spectinomycin) reduced significantly the bacterial growth but had also severe effects on the alga. The alga was moved to a fresh medium without antibiotics, but did not recover. In agar set-up shown in figure 7 the harmful antibiotics were added to a small portion of the agar that the filaments growing towards the light had to cross. The idea was that bacterial growth would be limited both by the agar and antibiotics more than the algal growth and that the filaments could recover in the antibiotic free top agar that had also access to light. In this experiment all algae died.

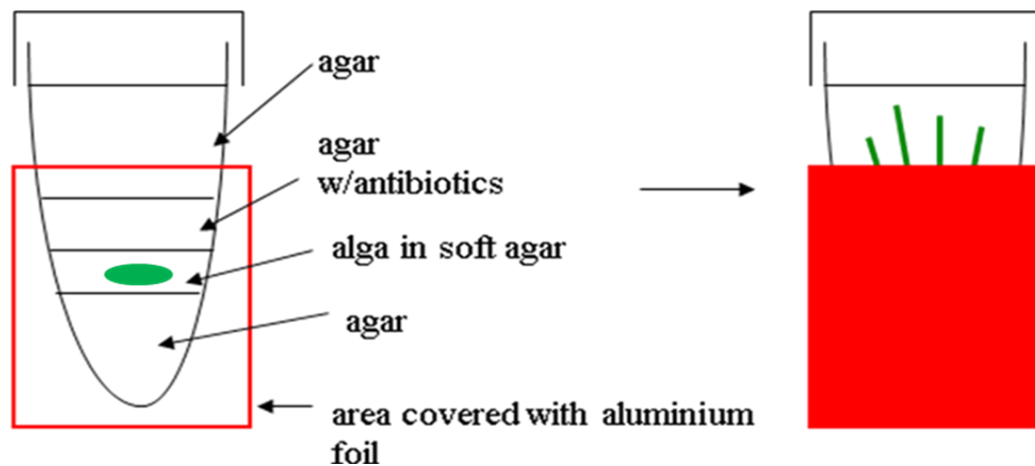


Figure 8: Agar set-up for removing the bacteria using phototropism combined with antibiotics. The bottom of a reagent tube was filled with medium solidified with 1% agar up to 2 cm height. Few filaments of the alga embedded in 0,5% agar-medium were immobilized between this layer and a 1cm thick layer of 1% agar-medium with antibiotics. The layer with antibiotics was further covered with antibiotic-free 1% agar-medium up to several cm and the bottom part of the tube was covered with aluminium foil. Antibiotics used: kanamycin, gentamycin, spectinomycin and chloramphenicol, in concentrations 0,2 mg/ml and 0,4 mg/ml.

3.2 Optimization of a protocol for chloroplast isolation from *Vaucheria*

There were two steps of the original protocol for isolation of chloroplasts from *V. litorea* as described in Green et al. (2005) that were adjusted: homogenization using a Polytron and filtration through a 110 μ m nylon net. The homogenization did not have effect on the small amounts of cut alga when larger volume of homogenization buffer was used. In smaller volumes, the homogenization was too destructive for the chloroplasts. Instead the small amounts of filaments were gently grinded in successive steps in small volumes of homogenization buffer with mortar and pestle. The homogenate was filtered through 45 μ m and 38 μ m sieves immediately after the filtration through cheesecloth to reduce the contamination already in the first centrifugation step. The filaments were also rinsed in several washes directly before the chloroplast isolation.

3.3 Structural stability of isolated chloroplasts

More than 80% of the chloroplasts isolated from *V. litorea* were intact immediately after isolation estimated by phase-contrast microscopy. The brightness declined only little in 24 h after isolation and more than 20% of chloroplasts appeared still intact 3 days after isolation (fig. 10). The chloroplasts of *V. bursata* never showed the same rate of intactness directly after isolation but the percentage of the bright chloroplasts remained also stable the first day after isolation and declined more rapidly the following days (fig. 10). In this study the chloroplasts of *P. sativum* never reached more than 30% of intactness after isolation but they stayed stable the first 4 h after isolation and 10% of chloroplasts still remained bright 24 h post-isolation time (results not included in the figure).

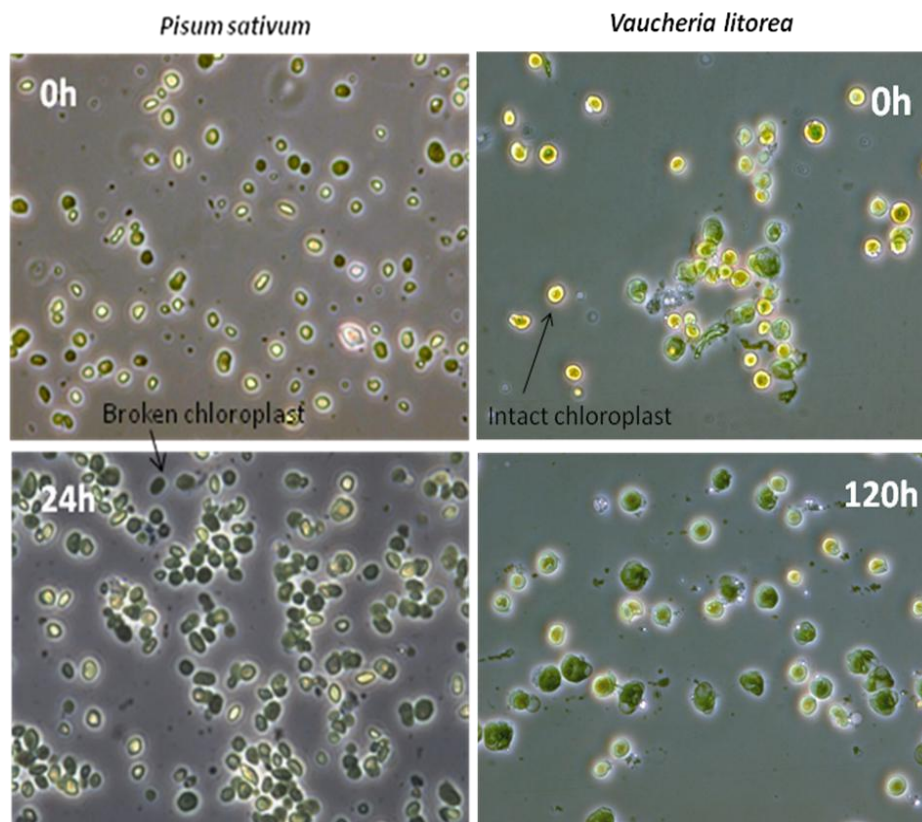


Figure 9: Intactness of isolated chloroplasts from *P. sativum* and *V. litorea* observed with phase contrast microscopy and 400x magnification immediately after isolation (0h) and one day after isolation (24h). Between the time points, chloroplasts were stored at 15°C and dim light. Intact chloroplasts appear bright and surrounded by halo, broken chloroplasts are dark with weak or absent halo.

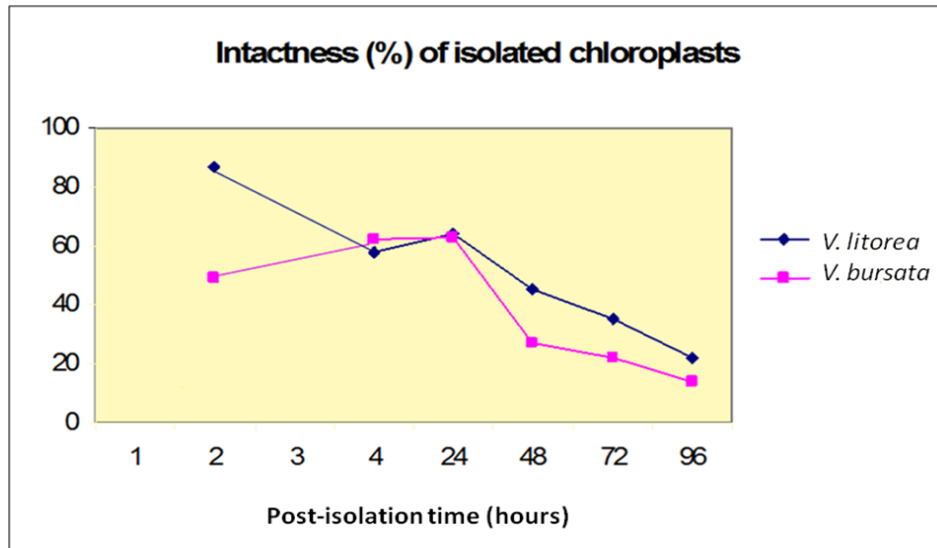


Figure 10: The percentage of intact chloroplasts isolated from *V. litorea* and *V. bursata* at different time points after isolation based on phase contrast microscopy. The percentage was calculated from the number of bright chloroplasts with halo against the total number of isolated chloroplasts estimated with Thoma counting chamber.

3.4 Analysis of overall transcriptional activity in isolated chloroplasts over time

The activity measured with the amount of ^{33}P -UTP incorporated into transcribed RNA (fig. 11) by 10^6 chloroplasts remained stable over 24 hours period in case of *V. litorea*. Chloroplasts of *P. sativum* showed stable transcriptional activity the first 4 hours and then transcription declined linearly until 24 hours after isolation. The transcription activity of *V. bursata* was more variable and increased significantly between 6 and 24 hours after isolation. The phase contrast microscopy confirmed presence of bacteria in the solution with isolated chloroplasts from *V. bursata*.

The results from two independent run-on transcription experiments measuring the overall transcriptional activity in isolated chloroplasts from *P. sativum* were compared in figure 12. The results were consistent with each other; both ^{33}P -UTP and ^{32}P -CTP were incorporated at the same rate and approximately in the same amount. The initially transcriptionally stable pea chloroplasts ceased gradually to incorporate the labeled nucleotides 4 hours after isolation.

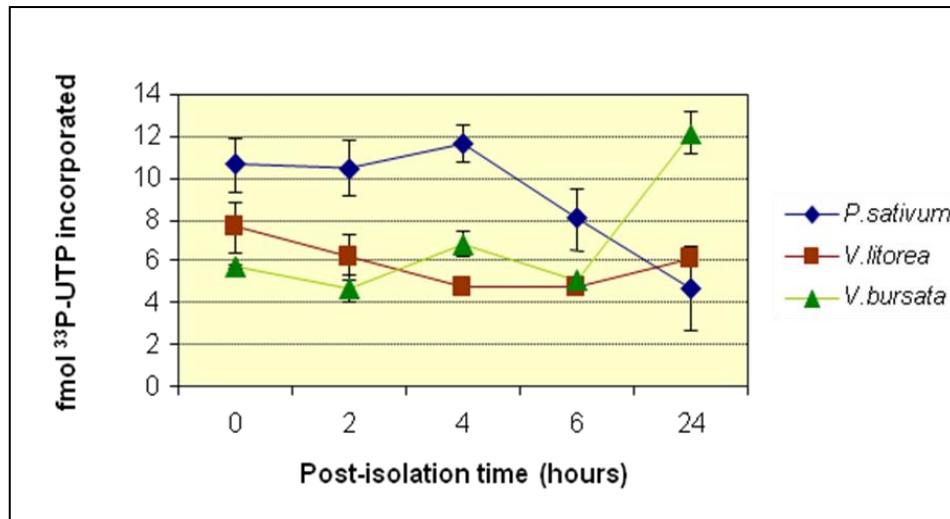


Figure 11: Run-on transcription assay - the amount of ³³P-UTP incorporated into transcribed RNA by 10⁶ of isolated chloroplasts of *V. litorea*, *V. bursata* and *P. sativum* at different time points after isolation. Each reaction was carried out for 6 minutes at 30°C. Radioactivity was measured in counts per minute (cpm - number of atoms in a certain amount of radioactive material that disintegrate in one minute) and the amount of ³³P-UTP incorporated was calculated using 1µl of unwashed reaction solution as an estimate of the cpm value for the total amount of ³³P-UTP (1,66 pmol) used in each reaction.

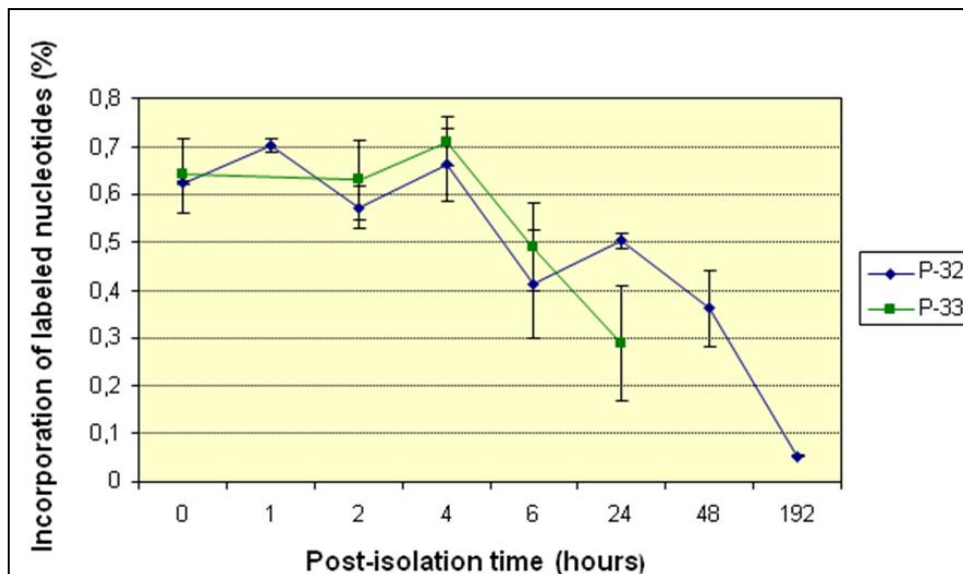


Figure 12: Run-on transcription assay - relative incorporation of radioactively labeled nucleotides into transcribed RNA by 10⁶ of isolated chloroplasts from *P. sativum* at different time points after isolation; comparison of results from two separate run-on assays using ³²P-CTP and ³³P-UTP, respectively. Each reaction was carried out for 6 minutes at 30°C. The percentage of incorporated radioactivity was calculated using cpm_s from 1µl of unwashed reaction solutions as the absolute values.

The hybridization of transcribed RNAs with probes targeting the selected genes immediately after isolation (0h) and 4 h after isolation is shown in fig. 13. The concentration of chloroplasts used in the labeling step was 2,5x lower for *V. litorea* than for *P. sativum*, so the intensity of the signal directly cannot be used for comparison between these two organisms for the amount of the specific RNA synthesized. Even though, the image of the radioactive signal from the hybridization membrane (fig. 13) shows clearly that in both cases the tRNA, rRNAs and *psbA* were synthesized at highest rate (weak but still visible signal for *V. litorea*). The difference between pea and *Vaucheria* lies in the intensity of signal between the *rrn23* and *rrn16*, with *Vaucheria* synthesizing more of the small rRNA and pea vice versa. Hybridization signals for both *V. litorea* and *P. sativum* show actually increase in RNA synthesis 4 hours after chloroplast isolation, rather than decline.

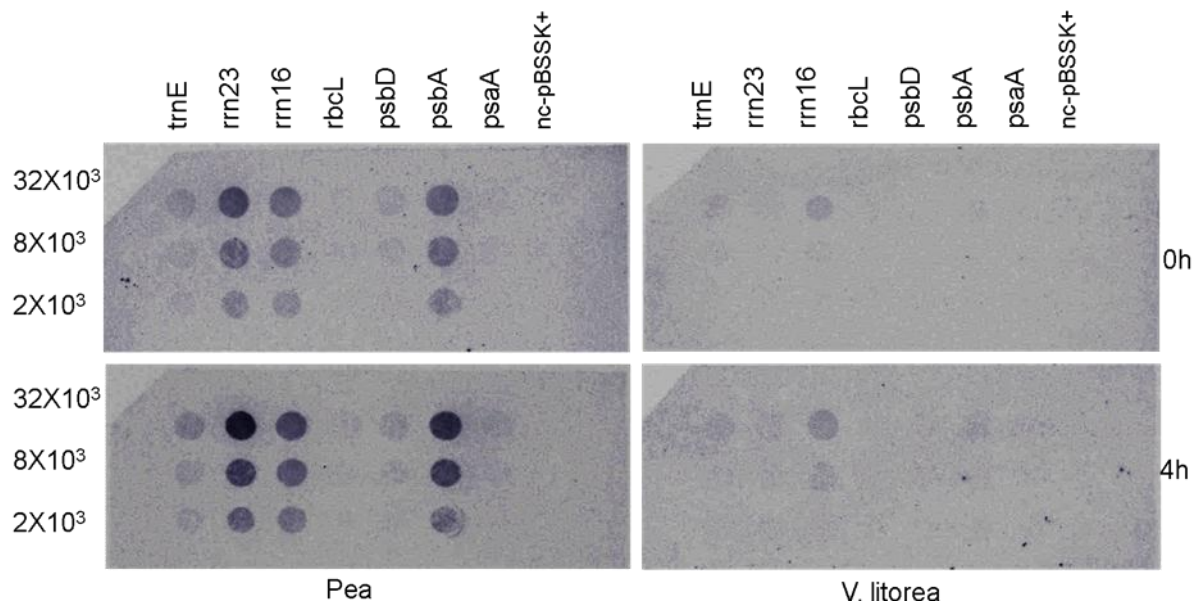
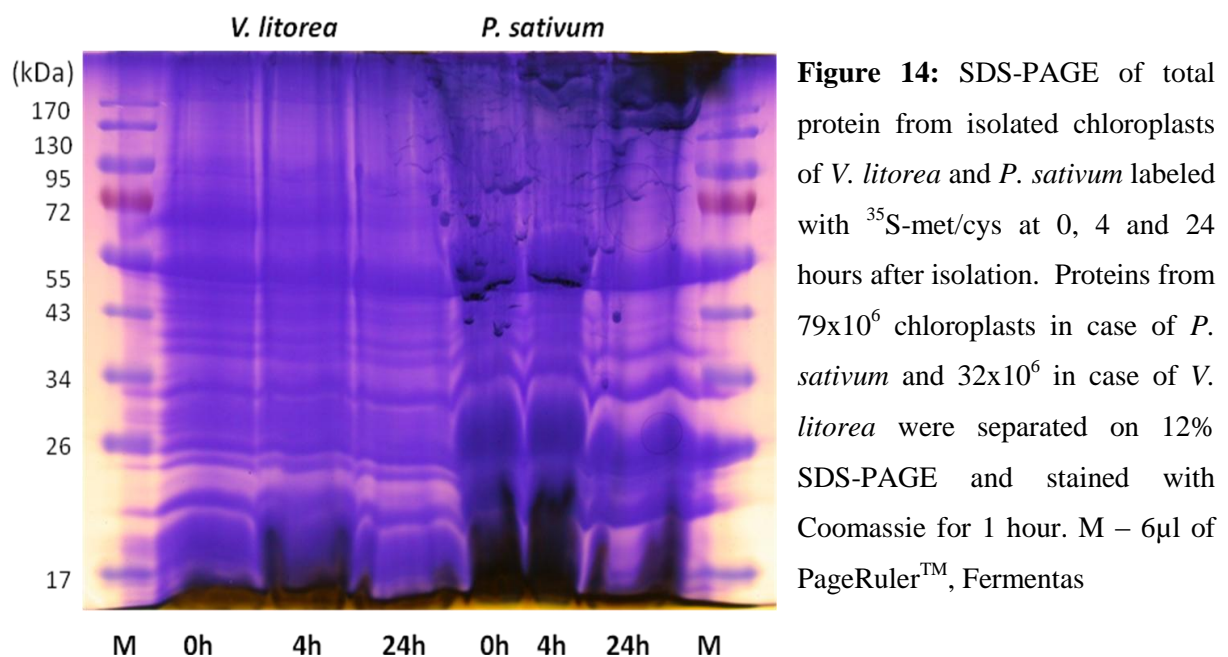


Figure 13: Dot blot hybridization of run-on transcripts labeled with ^{33}P -UTP from chloroplasts of *P. sativum* and *V. litorea* immediately after isolation (0h) and after 4 hours (4h). Between the time points, chloroplasts were stored at 15°C and dim light. Hybridization signals were detected by Personal Imager FX after 5 week exposure of the dot blot membranes to a phosphoscreen-K. Oligonucleotide probes were applied to the membranes in 1x, 4x and 16x concentration dilutions (1x: 32×10^3 fmol, 4x: 8×10^3 fmol and 16: 2×10^3 fmol). Nc-pBSSK+ is a short oligonucleotide specific for Bluescript SK+ and used as control for detection of unspecific binding. The concentration of plastids in 100µl of run-on reaction used for further hybridization was 2×10^7 in case of pea and 8×10^6 (2,5x less) in case of *V. litorea*.

All ribosomal genes are cotranscribed from one operon also in *V. litorea* (chapter 1.6.3) and the large and small subunits of ribosomes are assembled in stoichiometric manner (Rapp et al., 1992), so the discrepancy in transcription of 16S rRNA and 23S rRNA detected in the hybridization assay (fig. 13) was most likely due to differences in affinity of the DNA probes used.

3.5 Analysis of translational activity over time

The coomassie stain of the total protein (both labeled and non-labeled, fig. 14) isolated from the chloroplasts of *V. litorea* and *P. sativum* at 0, 4 and 24 hours post-chloroplast isolation time doesn't show a change in the amount of protein present in case of *V. litorea*. Samples at 0 and 4 hours after isolation in *P. sativum* were overloaded and the proteins were not separated efficiently. Therefore, it is difficult to estimate changes in the protein amounts in the samples, even though it appears that there was less protein present in the 24 hours sample of *P. sativum* compared to 0 and 4 hours samples (fig. 14). Both in samples from *V. litorea* and *P. sativum* there is no apparent overall change in band patterns of total protein present in the chloroplasts at each time point after isolation.



The isolated chloroplasts from *V. litorea* show rather stable incorporation of ^{35}S -met/cys between 0 and 4 hours after isolation (fig. 15). Also the band pattern is the same suggesting that the same proteins were synthesized at the same rate. The radioactive signal is much stronger in 24h post-isolation time sample from *V. litorea*, implying that there were more *de*

novo proteins synthesized, even though the amount of total protein stained with coomassie appeared to be the same at each time point (fig. 14). The band pattern in the upper area of the 24 h sample from *V. litorea* (marked with < in fig. 15) shows that new proteins with higher molecular weight were translated at this time point. Also, the radioactive signal is stronger in samples from *V. litorea* compared to pea samples even though the amount of chloroplasts used for protein labeling reaction was 2,5x less.

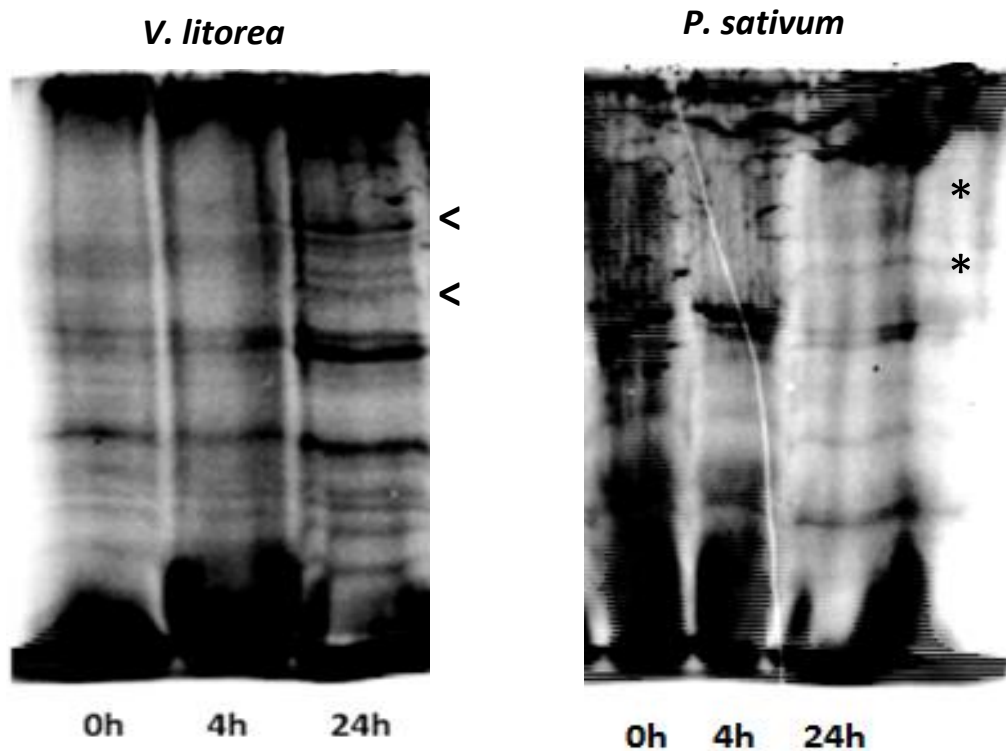


Figure 15: *In organello* translation of proteins in chloroplasts isolated from *V. litorea* and *P. sativum* labeled with ^{35}S -met/cys at 0, 4 and 24 hours after isolation. Each reaction was carried out at 28°C for 20 min., using 79×10^6 chloroplasts in case of *P. sativum* and 32×10^6 chloroplasts in case of *V. litorea* and 50 μCi of ^{35}S -met/cys. Total protein was separated by SDS-PAGE, gel was exposed to phosphoscreen-K for 7 days (*V. litorea* and *P. sativum*) and 14 days (*P. sativum* 14d exp.) and radioactivity was detected by Personal Imager FX.

Based on the signal intensity from samples of *P. sativum* it appears that chloroplasts perform still significant *de novo* protein synthesis 4 hours after isolation (fig. 15). The signal from 24 h sample of pea is weaker than at 0 and 4 hours but due to the consistency in intensity with the coomassie stain (fig. 14) it could also be caused by an unequal amount of chloroplasts in the 24h sample compared to 0 and 4 hours. As in case of *V. litorea* there is also change in the band pattern in 24 h sample with new proteins of higher molecular weight being synthesized (marked with * in fig. 15) at this time point.

3.6 Construction of a reporter construct for *in organello* transformation and protein expression

The primers used for amplification of the promoter sequence for *psbA* of *V. litorea* and *P. sativum* chloroplast (*ppsbA*), the sequence for *E. coli* ribosomal RNA operon T1 terminator (*rnnB* T1) and the enhanced green fluorescent protein (*egfp*) were designed to be flanked with sites recognized by specific restriction endonucleases in order to be excised from the helper pGEM vector and cloned into the final pBS SK+ vector. Their specificity is outlined in chapter 2.1.4, table 3. The *ppsbA* sequences were amplified from whole leaf pea DNA and from *V. litorea* chloroplast DNA, *rnnB* T1 from general cloning vectors with pENTR, D-TOPO and UNI51 backbones and *egfp* from the gateway cloning vector pB7FWG2. The fragments of expected sizes (fig. 16) were purified from the gels and concentrations measured with NanoDrop (table 15).

Table 15: concentrations of purified fragments after PCR

Fragment	Concentration
<i>ppsbA</i> pea	22,1 ng/μl
<i>ppsbA</i> V. l.	27,8 ng/μl
<i>rnnB</i> T1	52,2 ng/μl
<i>egfp</i>	52 ng/μl

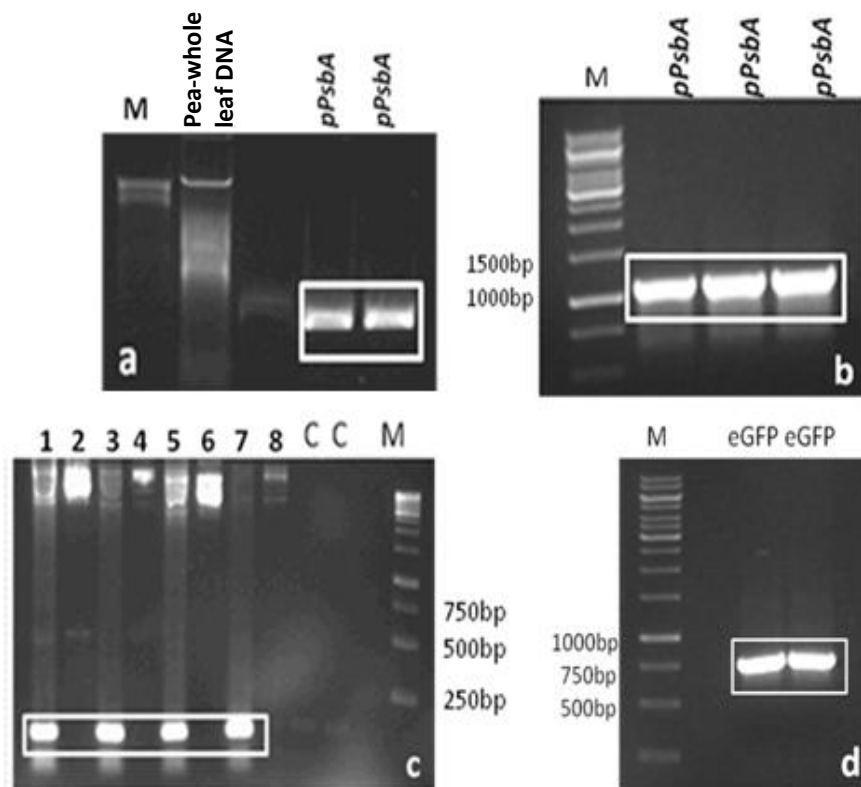


Figure 16: PCR amplification of fragments used for construction of a reporter construct, marked fragments cut out of the gels and purified: a – pea promoter for *psbA* (*ppsba*) from whole leaf DNA using *ppsba*-pea fwd and *ppsba*-pea rev primers, expected size 424bp; b - promoter for *psbA* from *V. litorea* chloroplast DNA using *ppsba*-V.l. fwd and *ppsba*-V.l. rev primers, expected size 1120bp; c - *rnnB* T1 terminator from plasmids with pENTR cloning vector backbones C105220 (lane 1) and U15884 (lane 3) D-TOPO backbone plasmid U15978 (lane 5) and pUNI51 backbone plasmid U67823 (lane 7), using T1 fwd and T1 rev primers, expected size 120bp, lane 2, 4, 6 and 8 – plasmid DNA of C105220, U15884, U15978, U67823, respectively; d – *egfp* from plasmid pB7FWG2 using *egfp* fwd and *egfp* rev primers, expected size 741bp, M - GeneRuler™ 1kb DNA ladder (Fermentas),

After subcloning of the fragments into pGEM-T and transformation of chemically competent *E. coli* cells, 20 of the colonies for each fragment were selected for screening of insertion by colony PCR. Four of the plasmids with the insert from each screen were isolated and sequenced. Except for *egfp*, the fragments with correct sequences were excised with the specific restriction enzymes and purified from gels after gel electrophoresis (fig. 17). The concentrations of the purified restriction fragments were 16,2 ng/μl for *ppsba* V. l. (measured with NanoDrop) and 1,5 ng/μl for *rnnB* T1 (estimated by comparison with standard molecular

weight ladder). There was no detectable DNA after purification of the pea *ppsba* fragment and the *egfp* was not excised from the pGEM-T due to the sensitivity of XbaI enzyme (restriction site for XbaI flanking the 3' end of *egfp*) to methylation. The DNA concentration of the entry vector pBS SK+ digested with NotI and SacI (fig. 17), enzymes also specific for *rnnB* T1, dephosphorylated and purified from the gel was 37 ng/μl.

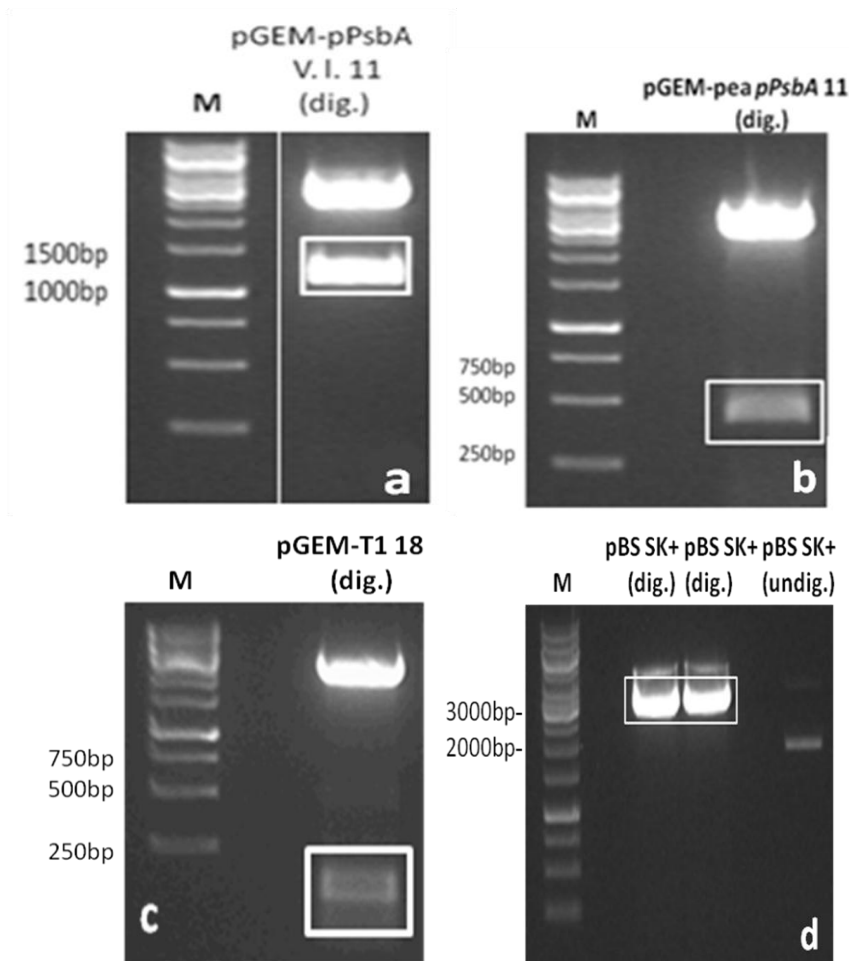


Figure 17: Digestion reactions of pGEM-T vectors with subcloned fragments and of entry vector pBS SK+, marked fragments cut out of the gels and purified: a – excision of *ppsba* V. I. from pGEM-T with HindIII and BamHI, 2 h double digestion, expected fragment size 1119bp; b – excision of pea *ppsba* from pGEM with HindIII and BamHI, 2 h double digestion, expected fragment size 424bp; c – excision of *rnnB* T1 from pGEM-T with NotI and SacI, overnight double digestion, expected fragment size 120bp; d – 2 h double digestion of pBS SK+ with NotI and SacI, expected fragment size 3kb, M - GeneRuler™ 1kb DNA ladder (Fermentas)

The digested *rnnB* T1 fragment was ligated with the digested pBS SK+ vector and the plasmids with the insert were detected by colony PCR after transformation into *E. coli*. The *egfp* with the correct sequence was amplified from the pGEM-T nr. 9 with PCR, using M13 pGEM-T specific primers. The *egfp* fragment extended with 116bp at the 5' end and 120bp at the 3' end was then digested in two steps with BamHI and XbaI, DNA purified from the gel (c with NanoDrop = 21,3 ng/μl) and ligated to the pBS SK+ with correct *rnnB* T1 insert, also digested with BamHI and XbaI and dephosphorylated (c with NanoDrop = 65,5 ng/ul). After transformation into *E. coli* and colony PCR, none of the 64 screened colonies contained the correct insert.

4. Discussion

The structural intactness and stability of isolated chloroplasts has been mainly studied in connection with the research on the photosynthetic function of these organelles. Since the beginning of 1930's, when Robert Hill and his coworkers started the uneasy task, the isolation of intact chloroplasts has been an ongoing rout of trial and failure. In the process of isolation from the “protective cocoon” (Walker, 2003) of the leaf, the chloroplasts must endure the rigorous mechanical forces, are exposed to the contents of vacuole and abrupt change in osmotic pressure and pH. To limit the damage, the transition step must be fast and the new artificial medium must resemble the natural environment as close as possible (Walker, 2003). The developed methods and media used had to be adjusted to reflect the requirements of the particular plant or algae species. For example, the mechanical isolation directly from leaves, that give high yields of intact chloroplasts from pea and spinach, cannot be applied on species with tough thickened tissues containing silica such as wheat or barley. The excessive force necessary to disrupt the cell walls also destroys the chloroplasts. In these species, the outer protective tissues must be first removed enzymatically to obtain protoplasts which then can be gently disrupted to release the organelles (Tobin, 1996).

The chemical requirements for chloroplast isolation are substantially more complicated than the mechanical ones. To obtain the optimal conditions for experiments with isolated chloroplasts, the specific isolation and storage media used in chloroplast preparations must not only be tested for the particular species but also for the particular application the isolated chloroplasts are prepared for. The chloroplasts from *Arabidopsis thaliana* show optimal stability in media with higher osmotic value than reported for pea or spinach and require low concentrations of $MgCl_2$ in the assay medium for photosynthetic reactions (Seigneurin-Berny et al., 2008). In opposite to *Arabidopsis*, free Mg^{2+} , added into the medium as $MgCl_2$ salt, inhibits photosynthetic reactions in pea, but in low concentrations stimulates the protein synthesis by isolated chloroplasts from this species (Nivison and Jagendorf, 1984; Piazza and Gibbs, 1983).

As the early research with isolated chloroplasts focused mainly on photosynthesis, the ability of isolated chloroplasts to evolve O_2 and to assimilate CO_2 was also originally used as a parameter for their structural intactness. This can be, as already mentioned, misleading due to

the diverse influence of certain compounds present in the media on different photosynthetic processes. Also, even though the ruptured chloroplasts can not assimilate CO₂ and perform CO₂ dependent O₂ evolution, they can still, in presence of oxidants and cofactors, produce O₂ and phosphorylate ADP to ATP. The other disadvantage of photosynthesis as a measure of intactness *in vitro* is that the depletion of P_i and accumulation of O₂ and ATP in the medium results in a quick decline in the activity even in intact chloroplasts (Cerovic et al., 1987). There are also cases where apparently structurally intact chloroplasts do not exhibit any photosynthesis at all, as with chloroplasts mechanically isolated from *M. perennis* (dog's mercury) (Lilley et al., 1975; Punnett, 1959).

In most cases, the structural intactness of isolated chloroplasts must be established prior to the experimental application. The structural intactness can be determined independently of the functional activity by phase contrast microscopy. When observed with phase contrast microscopy, chloroplasts can either appear bright with a light circle around referred to as a halo or dark, slightly larger, granulated and without halo. Supported by the fact that the bright chloroplasts turned dark when moved to hypotonic medium and further proved by electron microscopy, the bright chloroplasts were identified as intact with double-membraned envelopes surrounding the stroma content (Walker, 1965). Unfortunately, using chloroplast brightness and halo as intactness indicator can also represent some problems. The evaluation of brightness is subjective. In most chloroplast preparations, there are light and halo intermediates that can be misinterpreted. When estimating the ratio of intact and broken chloroplasts, usually only few counts with a dilution of a small sample portion are applied. To avoid errors, it is important to make a larger number of counts which is time consuming. Also, chloroplasts are fragile and tend to break more easily during the microscopy procedure (Lilley et al., 1975). The chloroplast envelope has also the ability to reseal itself after the disruption during isolation. Chloroplasts then retain the bright appearance even though some of the stromal components have been lost and the activity became impaired (Reith and Cattolico, 1985).

As the protocols of chloroplast isolation have been improved and established for diverse species, the research focusing on chloroplasts has, besides photosynthesis, extended to investigate other organellar processes (Clausmeyer et al., 1993; Ogrzewalla et al., 2002; Roy et al., 1982). However, due to the instability of isolated chloroplasts mentioned in the introduction, chloroplasts have not traditionally been used for direct *in organello*

transformation and gene expression experiments (Maliga, 2004). In one of the first plastid transformation experiments Daniell and McFadden (1987) documented that etioplasts isolated from cucumber cotyledon leaves and treated with EDTA can take up plasmid DNA from the solution. These etioplasts were also able to express the bacterial genes coding for antibiotic resistance. Activity of the enzymes was detected in chloroplasts that were lysed after 2 hours of incubation with plasmid DNA and 2 hours transcription-translation reaction. When the same experiments were carried out with plastids isolated from the plants at different stages of greening, Daniell and McFadden reported dramatic decrease in translation of the transformed gene in plastids from increasingly matured tissues. The electroporation method was first used by To and his colleagues in 1996. In their experiment the transformed spinach chloroplasts were incubated at dim light and 25°C for up 16 to 18 hours. The expression of the foreign genes was verified by protein isolation and detection of the expected enzyme activity (To et al., 1996). The electroporation method was further used to determine the DNA sequences necessary for replication in tobacco chloroplasts (Lugo et al., 2004) but in their study the degradation of introduced DNA started already 45 min. after transformation.

4.1. Stability of transcription and translation in isolated chloroplasts of *Vaucheria litorea*

4.1.1 Transcriptional activity

Results from run-on transcription assays show that RNA polymerase complexes are stably attached to DNA and, provided with all necessary compounds such as nucleotides and Mg^{2+} , can transcribe genes even 24 hours after chloroplasts isolation. Run-on transcription assays detect only the on-going transcription at the time of the reaction because heparin, used to stimulate the RNA polymerization and to minimize degradation of *de novo* synthesized transcripts (Klein and Mullet, 1990; Coupar and Chesterton, 1977), inhibits the initiation step. Not only heparin but also nucleotides necessary for the RNA synthesis are poorly transported across the envelope into intact chloroplasts (Deng et al., 1987) so run-on transcription reaction must be performed on lysed plastids. Therefore, this reaction does not differentiate between the chloroplasts broken directly prior to incubation or stable RNA polymerase-DNA complexes already released into the solution from previously disintegrated plastids. The verification of transcriptional activity in isolated chloroplasts was nevertheless a necessary first step in the investigation of the plastid activity and the ability to express genes several hours after isolation.

Based on the signals from the hybridization of the labeled RNA molecules to the DNA probes, the specificity of the genes transcribed and their comparable transcription rate did not change in the isolated chloroplasts over the 4 hour period. It was expected that *psbA*, coding for the D1 reaction centre protein of the PSII, would be among the most strongly transcribed genes. This protein is very susceptible to photodamage and it has the highest turn-over rate of all photosynthetic proteins in all species (Kettunen et al., 2004). In addition, due to their function as essential constituents of the chloroplast ribosomes, ribosomal RNAs are also strongly transcribed genes (Rushlow et al., 1980). In case of D1, previous studies have shown that the transcription of *psbA* is enhanced in response to light stress but in normal conditions the expression is rather controlled by differential mRNA stability and translation activity (Mullet and Klein 1987, Baumgartner et al., 1993). The transcription of transfer RNA (tRNA) genes is also generally high (Deng et al. 1987) but depends both on the codon usage and position in the genome (Rapp et al., 1992). The differential expression of tRNAs in chloroplasts from *V. litorea* has not been investigated yet, but in the plastid genome the *trnE* gene is located at the end of the gene cluster coding for ribosomal RNAs. The high transcription levels of this gene compared to *psbA* (fig. 13) may be caused by co-transcription with other highly transcribed genes.

Surprisingly, the transcription rate of the genes investigated, based on the hybridization results, has slightly increased from 0 to 4 hours after chloroplast isolation. This could be biased information, caused by increased amount of chloroplasts applied in 4 hour labeling reaction or the hybridization reaction. But, the increased hybridization signal for *V. litorea* is consistent with increased hybridization signal for *P. sativum*. Eberhart and colleagues reported that transcription of photosynthetic genes, such as *psbA* and *rbcL*, is up-regulated in chloroplasts of *Chlamydomonas reinhardtii* grown in suboptimal photosynthetic conditions (low light, impaired access of CO₂, rich growth medium) (Eberhart et al., 2002). There is a possibility that either the activity directly after isolation was impaired due to the mechanical stress and then stabilized until the following measurement, or the transcriptional increase was a chloroplast response to the stress of being separated from the alga and kept in pour light conditions. According to Doran and Cattolico (1997) there has not been reported any post-transcriptional RNA processing in chromophytic algae, the group that also includes *V. litorea*. They suggest that gene expression in these algae is regulated only at transcriptional level. It was also shown that chloroplasts of chromophytic algae contain sequences for a regulatory

protein that interacts with the sigma subunit of the plastid-encoded RNA polymerase and regulates its activity depending on the redox state in the chloroplasts (Duplessis et al., 2007).

4.1.2 Translational activity

In the present study, the translational activity of chloroplasts isolated from *V. litorea* and kept in artificial conditions remained stable for 24 hours. The stability and robustness of chloroplasts from coenocytic algae in general (Grant and Borowitzka 1984; Reith and Cattolico, 1985) but especially of those found in kleptoplastic associations with sacoglossan molluscs (Trench and Ohlhorst, 1976) was described previously, but transcriptional and translational activity of isolated chloroplasts over time was not tested until 2005 by Green and colleagues. They report unusual transcriptional stability with *de novo* synthesis of proteins unchanged for 3 days and the results presented in this study confirm their findings.

In general the mRNAs of higher plants and green algae are known to be rather long-lived (Zerges 2000). In barley the shortest half-life was reported for tRNA-Lys being 3 hours and the most stable mRNA was *psbA* with over 40 hours half-life (Kim et al., 1993). Both stability and translation are dependent on 5' and 3'-UTR and the secondary structures formed in these regions. The stem-loop structure at the 3'-UTR of *petD* mRNA (coding for subunit IV of cytochrome b_6f complex) in spinach chloroplasts prevents the degradation by exoribonucleases (Kudla et al., 1996). In chloroplasts of *C. reinhardtii* formation of stem-loop structures in the 5'-UTR mediates proper folding of *rbcL* mRNA which is required for accumulation and longevity of these transcripts (Suay et al., 2005). These stem-loop structures are also recognized by factors necessary for translational control of chloroplast gene expression. Experiments with nuclear mutants, also in *C. reinhardtii*, identified many of the nuclear factors needed for proper translation of plastid mRNAs, but the ability of isolated chloroplasts to sustain translation over time suggests that there are also intrachloroplastic translational signals (Trebitch and Danon, 2001; Link 2003 and 1996). One of the signals that alters both translation and stability of mRNAs of not only photosynthetic genes is light. Even though the transcription does not change significantly, the mRNAs for many of the photosynthetic proteins and also 16S mRNA accumulate in response to illumination (Klaff and Gruissem, 1991). The stability of many transcripts differentiates further during leaf maturation and growth of the plant in light, as best shown for *psbA*. The stability of mRNA decreases after the initial accumulation but the translation increases in light due to the ability of the translation factors to bind to RNA in reduced state (Marin-Navarro et al., 2007; Kim et

al., 1993). Unfortunately, none of these mechanisms in yellow-green algae (Zerges 2000) or the alteration in translation over time in isolated chloroplasts in general has been described previously.

In this study the overall protein pattern in the translational assay changed only slightly in the 24 hour sample (fig. 15). Two bands with rather strong radioactive signal appeared in the higher molecular weight area, which were not detectable in the earlier samples. One of the possible explanations is an initiation of expression or upregulation of proteins as a delayed response to the stress situation. In the chlorophytic alga *Haematococcus pluvialis* the up-regulation of plastidic ascorbate peroxidase, an enzyme that detoxifies hydrogen peroxide to water, does not occur until 24 hours after the oxidative stress induction (Wang et al., 2004). In the experiment of Green and colleagues the isolated chloroplasts were aliquoted by 25µl per tube and stored at 4°C in dark. In this project the final chloroplast solution was not aliquoted and it was kept at dim light and 15°C to imitate the natural conditions. The higher temperature used in this study allowed the chloroplasts to proceed with the processes also in between the reaction incubations. Even though the translation rate was not diminished in 24 hour sample and chloroplasts appeared also structurally and transcriptionally stable, expression of new proteins or up-regulation of already synthesized ones could be applied at this stage as a stress response. Another possibility is an impaired post-translational cleavage of *de novo* synthesized proteins. The D1 protein with a molecular mass of 32 kDa is produced from a 33,5 kDa precursor by post-translational cleavage of the carboxyl terminal end (Edelman and Mattoo, 2006). The new proteins observed in the 24 hour sample are of even higher molecular weight, suggesting that they were not a result of impaired cleavage of D1. However, depletion of factors from the solution after 24 hours of chloroplast incubation that are necessary for the processing could cause impaired cleavage of some other proteins and their appearance on the gel.

In his review on protein stability and degradation Adam (1996) describes that proteins synthesized in chloroplast that do not assemble into complexes are very sensitive to proteases and are rapidly degraded. It was shown that the plastid encoded chlorophyll binding proteins (CABs) are synthesized and targeted into the thylakoid membranes, but in the absence of chlorophyll they get rapidly degraded and do not accumulate. The same was observed with the large subunit of Rubisco (LSU, protein product of *rbcL*) in tobacco chloroplasts. In an experiment with a mutation in this protein that did not prevent its synthesis, only the assembly

into the holoenzyme, there was no detectable accumulation of either LSU or SSU (small subunit of Rubisco) in the organelles. The possible depletion of coenzymes or nuclear encoded subunits necessary for assembly of proteins into complexes could initiate this type of protein degradation in the plastids 4 hours after isolation and could account for the observed higher translational activity in the 24 hour sample compared to the overall protein content at this time point.

The general up-regulation of protein synthesis may be a stress response, as already discussed. However, if the gene expression in *V. litorea* is controlled mainly on the transcriptional level as described for chromophytic algae by Doran and Cattolico (1997), the increased overall translation could also be a result of an increased overall transcription. The transcription and translation in isolated chloroplasts of *Vaucheria litorea* would have to be further tested to verify the correlation in rate change over time.

4.2 Stability of transcription and translation in isolated chloroplasts of pea

4.2.1 Transcriptional activity

The activity of isolated chloroplasts from pea has been studied in more detail and in connection with more adverse applications than the chloroplasts of *V. litorea*. *P. sativum* is a traditional model organism in plant science and was used in the initial photosynthetic experiments with isolated chloroplasts (Walker 1965 and 2003) but further also in studies of chloroplast transcription (Tullberg et al., 2000), protein synthesis (Siddell and Ellis, 1975), protein transport (Fuks and Schnell, 1997) and many others. Even though the whole chloroplast genome of *P. sativum* as such has not been sequenced yet, many of the individual genes and additional sequences are already known (Sugiura 1992). Unfortunately, isolated pea chloroplasts showed rather limited photosynthetic (Punnett 1959) and translational stability (Nivison and Jagendorf, 1984). The transcriptional activity in isolated pea chloroplasts tested with run-on assays is readily used to investigate for example the gene expression in developing plants (DuBell and Mullet, 1995) and in response of the plant to different light treatment (Tullberg et al., 2000), but the stability of the transcription itself over time in isolated chloroplasts has not been tested. The verification of the transcriptional activity in pea chloroplasts is an important step in determination of the reasons for the recorded impaired translational activity. According to the results from this study the chloroplasts of *P. sativum* retained stable transcriptional rate until 4 hours after isolation (fig. 12) and in this time period they show higher incorporation efficiency than chloroplasts of *V. litorea* (fig. 11). These

findings could suggest that the previously documented poor protein synthesis in isolated pea chloroplasts could be caused by impaired translation and not transcription. However, as discussed in chapter 4.1.1, the run-on experiments are performed with lysed plastids and the stable transcription does not necessarily have to correspond with functionality of intact chloroplasts. It might also be useful to verify if whole length transcripts and not only intermediates are synthesized under the conditions used.

The radioactive signal from the hybridization assay with pea chloroplasts was much stronger than in case of *V. litorea* chloroplasts. It was of course 2,5x more pea chloroplasts available for the labeling of the plastids for the hybridization assay as mentioned in the result part. But pea chloroplasts also showed higher general transcription rate in the run-on experiments and there the amount of plastids was equal for both *P. sativum* and *V. litorea*. Otherwise the hybridization pattern of labeled transcripts was the same as in samples of *V. litorea* with *psbA* and ribosomal RNAs as the most abundant transcripts. The hybridization pattern of the selected genes remained also unchanged between 0 and 4 hours after isolation. The lack of correlation between RNA levels and transcription rates in isolated chloroplasts has previously led to the conclusion that the gene expression in higher plants was controlled mainly post-transcriptionally (Sugita and Sugiura, 1996). Newer data indicate that also transcription in higher plants is tightly regulated especially in response to the photosynthetic conditions. The variable photosynthetic redox state in chloroplasts is reflected by redox state of the thiol (-SH) group of functional proteins involved in gene expression (Link 2003). This mode of action, initially discovered to control translation initiation in the green alga *Chlamydomonas reinhardtii*, has shown to be involved in transcriptional control of many genes in higher plants (Link 2003). As isolation of chloroplasts from the plant and further storage in un-replenished medium in light and at 15°C would represent a stress situation for the organelles, the response to such situation was not reflected in the changed transcription rate of the selected genes, other than a possible increase. The unchanged pattern after 4 hours also supports the assumption that the transcription might be performed by intact, transcriptionally active chloroplasts rather than by exceptionally stable but random DNA-RNA polymerase complexes.

4.2.2 Translational activity

In this study isolated chloroplasts from *P. sativum* perform still significant *de novo* protein synthesis 4 hours after isolation and weak but easily detectable synthesis 24 hours after isolation (fig. 15). Based on the coomassie stain of the total protein in the isolated pea chloroplasts at time 0, 4 and 24 hours after isolation, the protein amount was approximately equal in 0 and 4 hour samples and then decreased in the 24 hour sample (fig. 14). As discussed in chapter 4.1.2, some of the aspects of the protein degradation in plastids are already known. Experiments from other researchers with isolated pea chloroplasts specifically showed that incubation in light enhanced rapid degradation of LSU with the concomitant appearance of degradation products in the range of 45 – 32 kDa. In darkness, LSU stayed stable for 4 hours. Degradation of phosphoribulokinase (PRK, enzyme regenerating ribulose-1,5-phosphate in the final step of the Calvin cycle) did not proceed as quickly but the levels of PRK were still reduced significantly after 4 hours (Roulin and Feller, 1998). In this project no major decline in the general protein content was observed even 24 hours after isolation. The kinetics of the individual proteins has not been performed here due to the time limitations for the present study.

As the protein degradation in isolated chloroplasts is generally expected to proceed rather fast, the smaller unidentified fragments present in *in vitro* labeled samples have been assigned as degradation products. In the presented results, the smear appearance and poor resolution of the proteins is present especially in the 0 hour sample and in the lower molecular weight region of both the 0 and 4 hour sample (fig. 15). The absence of these unspecific fragments when chloroplasts were labeled *in vivo* and the disappearance of the smear fraction when the protocol for *in vitro* protein labeling was adjusted led Mullet and colleagues (1986) to the conclusion that those were translation intermediates produced by paused translation. Using antibodies binding to the N-terminal part of the LSU they showed that the signal for the full length protein of 55 kDa was increasing in intensity while signals from diverse fragments of smaller size were fading when the initial short (15 min.) labeling reaction with ³⁵S-met was followed by translational reaction with cold methionine prolonged from 5 to 10, 20 and 30 min. The mechanisms behind the apparent effect of the labeled methionine on the stalling of the translating ribosomes were not discussed in the publication. In translational experiments of Nivison and Jagendorf (1984) and of Siddell and Ellis (1975), pea chloroplasts were incubated together with the radioactive amino acid over the whole test period and only aliquots were taken for the incorporation measurements at each time point. It is possible that

in their experiments the translational activity was impaired by the experimental conditions but not by chloroplast instability itself. It would be interesting to test if more specific protein patterns with stronger signal also after 24 hours could be achieved by extending the translation reaction with incubation using cold methionine.

4.3 Aspects of culturing and chloroplast isolation with respect to *Vaucheria litorea*

There were three aspects connected with *V. litorea* that rendered this alga rather difficult to work with: contamination, slow growth, and instability of chloroplasts during isolation.

Marine algae are known to produce extracellular polysaccharides that stimulate growth and chemotaxis of motile bacteria. In the established mutualistic relationship, the excreted algal polymers are hydrolysed by the bacteria that in return provide the alga with minerals, organic nitrogen, phosphorus, and vitamins (Guerrini et al., 1998; Croft et al., 2005). This interaction establishes early in the algal growth and stimulation of bacteria can result in extended mineralization of the algal material. As the nutrients get depleted from the medium, bacteria will compete for the minerals. In these stress conditions, the alga produces even more polysaccharides, the surface gets sticky and is further colonized by bacteria, the photosynthesis is impaired and finally the alga dies (Guerrini et al., 1998). The associations of bacteria with the surface polymers of the alga explain the inefficiency of the attempts to obtain an axenic culture with the agar set-ups. It was also difficult to remove the bacteria completely during the chloroplast isolation. Even though they multiplied rather slow in the chloroplast solution, they made the chloroplasts less viable over longer incubation period and interfered with the run-on results as showed in fig. 11 with *V. bursata* 24 hour sample.

Not only the algal requirements itself but also the bacterial colonization cycle makes it important to change frequently the culture media and to remove the oldest filaments before re-inoculation. The large cultures were inoculated in 2,5 liter flasks, when trying to obtain larger amounts of the alga; these flasks had also larger space requirements in the growth chamber. The chloroplast isolation efficiency was low, 3 - 5 large 6 – 8 weeks old cultures usually represented only 2g of wet weight alga, rendering only 100µl of the final chloroplast solution with a concentration of $10^5/\mu\text{l}$.

Finally, the reported robustness of chloroplasts from *V. litorea* (Green et al., 2005) did not correspond with its susceptibility to get destroyed during the isolation procedure. Homogenization of the alga in the blender usually resulted in very low chloroplast yields. The

filaments had to be disrupted by gentle grinding in small portions at a time, extending the isolation duration and thus the stress situation for the chloroplasts. The time aspect of the efforts associated with culturing and isolation, resulting in only low chloroplast yields, would be the major disadvantage of *Vaucheria litorea* as a suitable donor of chloroplasts for further applications.

4.4 Conclusion

Chloroplasts of *Vaucheria litorea* show more stable protein synthesis than chloroplasts of *Pisum sativum*, extending for at least 24 hours after isolation. On the other hand, in this project chloroplasts of *V. litorea* did not show superior resilience to withstand mechanical disruption in comparison with pea chloroplasts. These chloroplasts have never been used for transformation and it is not known how stable an extraorganellar DNA would be in these plastids. Compared to *V. litorea*, *P. sativum*, including its chloroplasts, is a well studied organism. It has simple culturing requirements and rapid growth. Isolation of large amounts of uncontaminated chloroplasts from this plant is fast and both technically and financially undemanding. Even though the incorporation of radioactive labeling by pea chloroplasts was declining, the signal intensity was still significant after 4 hours and readily detectable even 24 hours after isolation. As the cleaning and up-scaling of the *Vaucheria* cultures would rather be a time consuming procedure, it is also important to test if the possible reduced activity of chloroplasts from *P. sativum* can be compensated for by increased concentration. The determination of the transformation rate and the ability to express foreign genes from introduced DNA will be an important step in order to decide which organism provides chloroplasts better suited for *in organello* assays. The plastid transformation vector with green fluorescent protein designed and partially constructed in this project will provide a good tool for this estimation. Besides conventional microscopy, the fluorescent signal can also be detected by a flow cytometer which can be used to obtain more accurate results.

5. References

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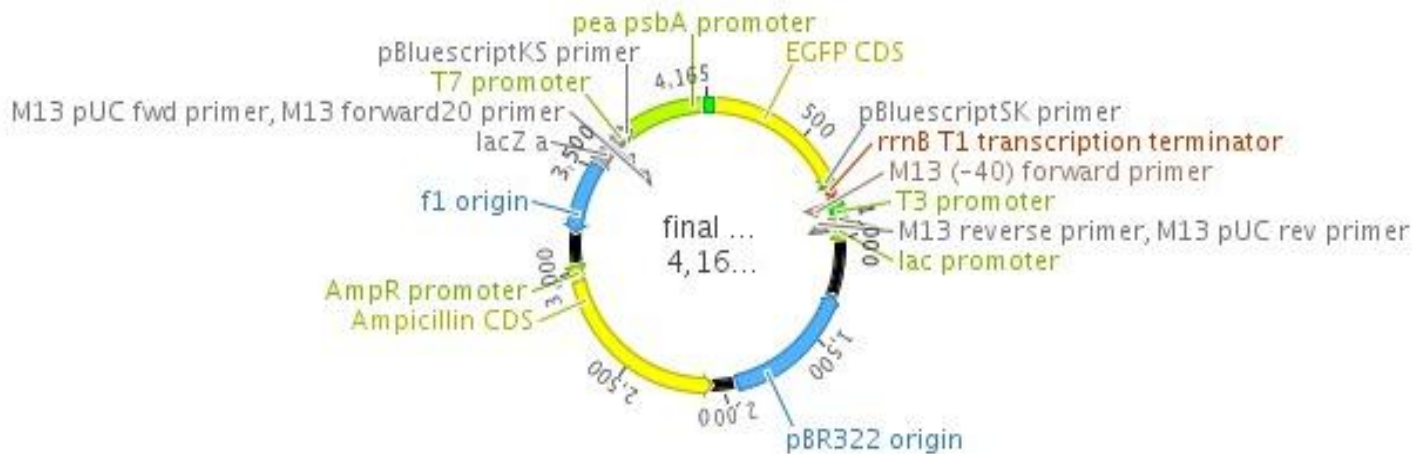
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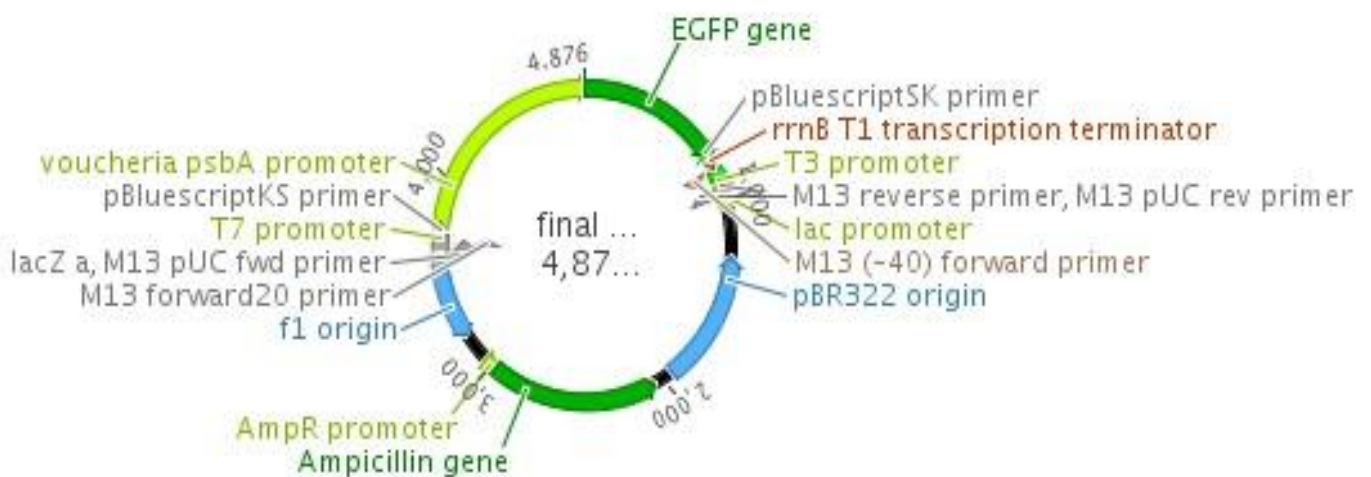
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Appendix

Gene maps of the chloroplast transformation vectors. The *egfp* sequence, coding for enhanced green fluorescent protein, with the respective *psbA* promoters and *rrnB* T1 terminator were cloned into MSC of pBluescript SK+ cloning vector.



A – transformation vector for *P. sativum*, final size 4 165 bp



B – transformation vector for *V. litorea*, final size 4 876 bp