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AKDENİZ ÜNİVERSİTESİ

ISOLATION, CLONING, CHARACTERIZATION and HETEROLOGOUS EXPRESSION in *Escherichia coli* of *Chlamydomonas reinhardtii* PYRUVATE ORTHOPHOSPHATE(Pi) DIKINASE(PPDK) 1 gene

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TARIMSAL BİYOTEKNOLOJİ (İNGİLİZCE)

ANABİLİM DALI

YÜKSEK LİSANS TEZİ

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ANABİLİM DALI

YÜKSEK LİSANS / DOKTORA TEZİ

Bu tez 02/07/2018 tarihinde jüri tarafından Oybirliği ile kabul edilmiştir.

Prof. Dr. Tarlan MAMMEDOV (Supervisor)
Prof. Dr. Yaşar KARAKURT
Dr. Ögr. Üy. Ersin AKINCI

ÖZET

Chlamydomonas reinhardtii PURİVAT ORTOFOSFAT(Pi) DIKINASE(PPDK) 1 geninin *Escherichia coli*'de KLONLANMASI,KARAKTERİZASYONU VE HETEROLOG EKSPRESYONU

Taha TANGUT

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı
Danışman: Prof. Dr. Tarlan MAMMEDOV
Aralık 2017, 30 sayfa

Fosil yakıt kaynaklarının sınırlı olduğu göz önüne alındığında, araştırmacılar alternatifler aramışlardır. Algler en umut verici alternatiftir ve geleneksel biyoyakıt potansiyeli olan bitkilerin üzerinde belirgin avantajlar sunar. Bununla birlikte, ökaryotik, tek hücreli, fotosentetik mikroalerde lipid metabolizması ile ilgili çalışmalar sınırlıdır. Chlamydomonas reinhardtii, on yıldan uzun süredir bir dizi fizyolojik, biyokimyasal ve genetik çalışma için model organizma olarak kullanılan ökaryotik mikroalglerin en iyi bir temsilcilerinden birisidir. Bununla birlikte, şaşırtıcı bir şekilde C4-metabolizması ve düzenleyici faktörlerin enzimleri, moleküler düzeyde C. reinhardtii'de incelenmemiştir, ki bu son çabalar dışında, fosfoenolpiruvat karboksilaz (PEPC) genlerinin (PPC 1 ve Ppc2) moleküler karakterizasyonu üzerine odaklanmıştır. C. reinhardii (Mamedov ve arkadaşları, 2005; Moellering ve diğ., 2007; Mamedov ve Chollet, 2010). Bu nedenle, fotosentetik model organizma C. reinhardtii'de C4-metabolizmasının enzimlerinin incelenmesi, alglerdeki biyokütle birikimi ve biyoyakıt için çok önemlidir. Piruvat, fosfat dikinaz (PPDK, EC 2.7.9.1), C4 bitkilerinde ve bazı POM bitkilerinde fotosentetik CO2 fiksasyonunun yolunda önemli bir enzimdir ve ATP ve Pi'ye bağlı fosfoenolpiruvat (PEP) oluşumunu katalize eder piruvattan alıcı molekül. Bu çalışmada, ilk kez, Chlamydomonas reinhardtii'nin cDNA'sından PPDK (CrPPDk) genini başarılı bir şekilde izole ettik ve klonladık. Sonuçlarımız, CrPPDK geninin in vivo olarak kopyalandığını ve E.coli'de tamamen aktif, rekombinant bir PEPC'yi kodladığını doğrulamıştır. Gen ekspresyonu analizleri, CrPPDK geninin CO2 veya NH4 + duyarlı gen olduğunu ve kararlı durum transkript seviyelerinin, büyüme ortamına sağlanan değişen CO2 veya NH4 + seviyeleri tarafından yukarı / aşağı düzenlendiğini göstermektedir.

ANAHTAR KELİMELER: Algal Biyodizel, *Chlamydomonas*, C4 Fotosentez, PPDK,CO2 fiksasyonu,gen ekspresyonu,biyoyakıt

JÜRİ: Prof. Dr. Tarlan MAMMEDOV (Danışman)
Prof. Dr. Yaşar KARAKURT
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ABSTRACT

ISOLATION, CLONING, CHARACTERIZATION and HETEROLOGOUS EXPRESSION in *Escherichia coli* of *Chlamydomonas reinhardtii* PYRUVATE ORTHOPHOSPHATE(Pi) DIKINASE(PPDK) 1 gene

Taha TANGUT

Master Degree Thesis, Agricultural Biotechnology Department
Supervisor: Prof. Dr. Tarlan MAMMEDOV
December 2017, 30 page

ABSTRACT

Given that fossil fuel resources are limited, the researchers look for alternatives. Algae is the most promising alternative and offers distinct advantages over traditional oil crops. However, studies on lipid metabolism in eukaryotic, single-celled, photosynthetic microalgae are limited. Chlamydomonas reinhardtii is a well-studied representative of eukaryotic microalgae that has been used as a model organism for a number of physiological, biochemical and genetic studies for more than a decade. However, surprisingly enzymes of C4-metabolism and regulatory factors have not been studied in *C. reinhardtii* at the molecular level, except the recent effort, which was focused on the molecular characterization of phosphoenolpyruvate carboxylase (PEPC) genes (Ppc 1 and Ppc2) in *C. reinhardii* (Mamedov et al., 2005; Moellering et al., 2007; Mamedov and Chollet, 2010). Thus, study of enzymes of C4-metabolism in the photosynthetic model organism *C. reinhardtii* would be very important for algal biomass accumulation, hence for biofuel. Pyruvate, phosphate dikinase (PPDK, EC 2.7.9.1) is important enzyme in the pathway of photosynthetic CO_2 fixation in C4 plants and in some CAM plants, which catalyzes the ATP- and Pi-dependent formation of phosphoenolpyruvate (PEP), the primary CO_2 acceptor molecule, from pyruvate. In this study, for the first time, we successfully isolated and cloned *PPDK (CrPPDk)* gene from cDNA of *Chlamydomonas reinhardtii.* Our results confirmed that CrPPDK gene transcribed *in vivo* and encodes a fully active, recombinant PEPC in *E.coli*. Gene expression analyses demonstrate that *CrPPDK* gene is CO_2 or NH_4^+ responsive gene and its steady-state transcript levels are up-/down-regulated by varying levels of CO_2 or NH_4^+ supplied to the growth medium.
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KEYWORDS: Algal Biodiesel, *Chlamydomonas*, C4 photosynthesis, C. *reinhardti,* PPDK, CO_2 fixation, gene expression, biofuel

COMMITTEE: Prof. Dr. Tarlan MAMMEDOV (Supervisor)
Prof. Dr. Yaşar KARAKURT
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PREFACE

I would like to express my sincere gratitude and appreciation to my supervisor Prof. Dr. Tarlan MAMMEDOV (who was like a father to me in every manner after the death of my own), my mom Fatma TANGUT, my brother Musa TANGUT and my late father Mustafa TANGUT (who will be forever loved) and other unsung heroes in my life for supporting me all the time. I also want to thank all researchers and my lab mates in our department for their help during my work. Thank you all for everything that you have done for me .

CONTENTS

AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum "Isolatıon, Cloning, Characterization and Heterologous Expression in *Escherichia coli* of *Chlamydomonas reinhardtii* PYRUVATE ORTHOPHOSPHATE(Pi) DIKINASE(PPDK) 1 gene" adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak yazıldığını belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

Tarih: 02 / 07 / 2018

Taha TANGUT

SYMBOLS AND ABBREVIATIONS

Symbols

µL	Microliter
µg	microgram
bp	base pair
min.	Minutes
G	Gram
Ha	Hectare
Kb	Kilobase
kDa	kilo dalton
Kg	Kilogram
L	Liter
mL	Mililiter
s	Second

Abbreviations

CRISPR	Clustred Regularly Inter Spaced Palindromic Regions
DNA	Deoksiribonucleic acid
OAA	Oksaloacetic acid
ORF	Open Reading Frame
qPCR	Quantitative Polimerase Chain Reaction
PCR	Polimerase Chain Reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate Carboccilase
PEPCK	Phosphoenolpyruvate Carboccicykinase
PPDK	Pyruvate Ortophosphate Dikinase
TALEN	Transcription Activator Like Endo Nuclease
TAG	Triachilglyserol
TAP	Tris Asetate Phosphate

FIGURES

TABLES

1. INTRODUCTION

As a result of increased industrialization and developing transportation networks, the need for fuel energy is also increasing. Against the ever-increasing need for fuel, our fuel resources are rapidly depleting. The researches conducted in 2009 showed that; even if the pace of consumption does not change, existing resources will be able to face the world's 42-year oil demand (Owen et al., 2010). Biofuels, which are renewable energy sources, have the capacity to be an alternative to fossil fuels, as they are both low in carbon emissions and continuously renewable. However, the production process of these fuels is very costly. As a result of the scientific studies carried out, the production cost of different biofuel species has been partially reduced and bioavailability has become comparable to fossil fuels (Puhan et al., 2005). Microalgae are of great interest in the search for a sustainable feedstock for the production of biofuels. However, the present cost of various algae species is typically between 5 and 10 US$ per kg dry weight; therefore, currently plant biologists are working on strategy to reduce the costs of production of algae so that the process becomes commercially viable.

Microalgae assimilate carbon dioxide (CO_2) as the carbon source for growth, thus can contribute significantly to the mitigation of atmospheric CO_2. One of the factors limiting CO_2 fixation in green algae is that Rubisco has a surprisingly poor affinity for CO_2. A number of photosynthetic organisms have developed ways to increase the level of CO_2 at the location of Rubisco in the plant. An excellent example of a CO_2-concentrating mechanism in higher plants is C4 photosynthesis (CO_2 fixation by C4 metabolic pathway), which are the best-studied organisms that concentrate CO_2 to enhance the carboxylation reaction of Rubisco. C4 photosynthesis metabolic pathways are essential for high rates of CO_2 fixation in plants. High rates of photosynthesis are important for high biomass accumulation of algae. *Chlamydomonas reinhardtii* is a well-studied representative of eukaryotic microalgae that has been used as a model organism for a number of physiological, biochemical and genetic studies for more than a decade. However, surprisingly C4 pathway enzymes and regulatory factors have not been studied at the molecular level, except our recent effort, which was focused on the molecular characterization of phosphoenolpyruvate carboxylase (PEPC) genes (Ppc 1 and Ppc2) in C. reinhardii (Mamedov et al., 2005; Moellering et al., 2007; Mamedov and Chollet, 2010). Thus, given the complete lack of molecular and biochemical insight into the enzymes of C4-metabolism and regulatory factors in C. *reinhardtii*, study of enzymes of C4-metabolism in the photosynthetic model organism C. *reinhardtii* would be very important for algal biomass accumulation. Furthermore, these studies would also be important to modify the levels of oil storage versus proteins storage in algal cells to get oil-rich algae. At the moment a little information is available for molecular mechanisms that control oil accumulation in microalgae. Thus, our long term research goal is focused on minimizing oil feedstock cost by engineering the C. *reinhardtii* metabolism to enhance the accumulation of triacylglycerols (TAGs) while maintaining efficiency of carbon dioxide conversion.The immediate goal of this project is detailed molecular and biochemical characterization of C4 pathway enzymes such as pyruvate, orthophosphate (Pi) dikinase (PPDK, EC 2.7.9.1) from C. *reinhardtii* cells. PPDK catalyzes the ATP- and Pi-dependent formation of phosphoenolpyruvate (PEP), the primary CO_2 acceptor molecule, from pyruvate. Thus, PPDK has a role in the altering the level of PEP in algal cells and high activity of PPDK may result in the use of high levels of pyruvate, which is

a major product for TAG synthesis. Thus, for the first time, we successfully isolated and cloned *CrPPDK* gene from cDNA of C. *reinhardtii*. Our results confirmed that *CrPPDK* gene produced in E. coli was fully active indicating that this gene encode functional active PPDK proteins in C. *reinhardtii* cells. Gene expression analyses of *CrPPDK* gene demonstrate this gene is CO_2 or NH_4^+ responsive genes and their steady-state transcript levels are up-/down-regulated by varying levels of CO_2 or NH_4^+ supplied to the growth medium. All these findings demonstrate that PPDK gene can be candidate for silencing the genes for possible oil increase in C. *reinhardtii* cells.

Various resources are used to produce biofuels. Algeas are used as third generation biofuels for several reasons. First of all some of these resources are source of food and used in diets. Secondly these raw materials do not give sufficient energy. Therefore algeas which are not a source of food and provide sufficient energy used for the production of energy. Algal cells naturally have a high level of triglycerol, which can be used for production of biodiesel. Although these characteristics of algal cells provide an opportunity to decrease biodiesel prices the cost of fuel production,molecular studies arre conducted to increase the yield of biofuel in algal cells. According to the Goncalves et al., by silencing various genes,they have successfully increased oil yield in *Chlamydomonas reinhardtii* which is a model organism and used for the microalgal studies (Goncalves et al., 2015).

Chlamydomonas cells exposed to nitrogen starvation have decreased expression of protein biosynthesis, TCA cycling and photosynthesis related genes, whereas expression of genes associated with TAG biosynthesis has been increased (Miller et al., 2010). It is not known exactly which genes need to be altered on the C4-like photosynthesis system in order to increase the oil ratio, since the C4-like photosynthetic system predicted to be found in *Chlamydomonas reinhardtii* is not yet fully characterized. Characterization of the PPDK1 enzyme in *Chlamydomonas reinhardtii* will help to clarify the C4-like photosynthesis system of a model organism and will also lead to studies on the development of highly cellular algae as in the PPDK1 enzyme.

2. LITERATURE

2.1. Microalgea

Microalgae are fast growing photosynthetic microorganisms that can be prokaryotic or eukaryotic, which can survive in difficult conditions. Cyanobacteria and green algae could be example for procaryotes, and diatoms could be example for eukaryotes (Li et al., 2008). Microalgae are found in all ecosystems on earth and can live in a variety of environmental conditions. It is estimated that there are more than 50 000 species of algea and about 30 000 of them have been analyzed (Wang et al., 2010). Microalgae are considered to be the third generation biofuels source because of their potential. Since they are a renewable and sustainable fuel source, they are being used as an alternative to fossil fuels in the solution of environmental problems such as carbon emissions reduction (Ho et al., 2014). Microalgae also has many advantages over other sources of biofuels. They have high photosynthetic efficiency, less space required for cultivation, and not food source like the other valuable source (Wijffels et al., 2010). Despite all these advantages, microalgae fuels have high production costs. Genetic improvement in microalgae strains has partially solved this problem, but still needs to be improved.

2.2. Oil Storage in Algae

The performance of oil yields obtained from algae is 200 times greater than the highest oil yielded plant (Table 2.1). Some algae can produce as much oil as half of its biomass (Chisti, 2007) (Figure 2.2). Naturally, the proportion of oil that they produce is high, and these ratios can be further increased under different conditions. Algae accumulate high amounts of neutral lipids by altering their metabolism under stress conditions (Grif fiths and Harrison, 2009; Griffiths et al., 2012; Markou and Nerantzis, 2013; Merchant et al., 2012). Atmospheric carbon and organic carbon sources that are linked by photosynthesis, which are to be used in the synthesis of primary metabolism products such as starch and protein during the absence of nitrogen, are directed to the synthesis of triacylglycerol (TAG) (Deng et al., 2010). As a result of many studies in algae, alteration of the direction of carbon flow blocking starch synthesis is an effective way to increase TAG synthesis. The TAG obtained is important in that it can be used as biodiesel after being subjected to various processes (Li et al., 2010; Ramazanov and Ramazanov, 2006; Wang et al., 2009; Work et al., 2010).

Table 2.1. Comparison of oil percentage, oil production, land use and biodiesel production of various agricultural products and microalgae (Mata et al., 2010).

	Oil content %	Oil production L/ha/year	Land use m^2.year/kg.b.d	Biodiesel production kg.b.d/ha.year
Maize	44	172	66	152
Soy	18	636	18	562
Canola (colza)	41	974	12	862
Sunflower	40	1070	11	946
Palm	36	5366	2	4747
Microalg (less)	30	58.700	0.2	51.927
Microalg (average)	50	97.800	0.1	86.515
Microalg (high)	70	136.900	0.1	121.104

Table 2.2. Comparison of oil content in dry weight of some microalgae species (Chisti, 2007).

Mikroalgea	**Oil content (dry weight %)**
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30

2.3. Model Organism *Chlamydomonas reinhardtii*

Green alga *Chlamydomonas reinhardtii* is a very suitable model organism for the investigation of photosynthesis, phototaxis, algal carbon concentration mechanism and algal lipid metabolism. Chlamydomonas has great similarities with plant kingdom in terms of plant origin and biological characteristics. The results of the investigations have been accepted as the result of this algae plant kingdom. It is also used extensively in biotechnological applications, such as the optimization of algal metabolic pathways for biofuel production and therapeutic protein production (Hannon et al., 2010; Jinkerson et al., 2011, Rasala and Mayfield, 2011; Scranton et al., 2015; Terashima et al., 2015).

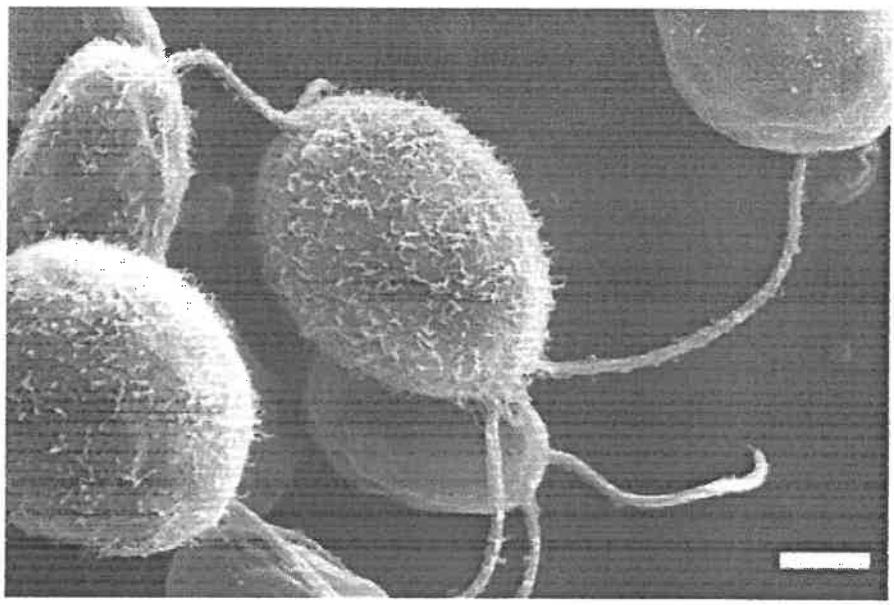

Figure 2.1. *Chlamydomonas reinhardtii* 3D view (Dartmouth College Electron Microscopy Center, 2015)

Some features of this organism make it a useful platform for many cases. Chlamydomonas can grow heterotrophically in the dark if it is fed phototrophically with acetate under light. The metabolic flexibility of this organism allows for the mutagenesis of the genes of the main mechanism of the photosynthesis system, which can be lethal for other organisms. Chlamydomonas is a single-celled structure (Fig. 2.2), despite the complexity resulting from high-pitched tissues. Liquid cell cultures can easily be grown in the laboratory and cell cycles can be synchronized in light / dark environments. Vegetative cells are haploid, and recessive mutations immediately manifest themselves in the phenotype. The haploid nuclear genome is approximately 111.1 Mb (Blaby et al., 2014). Chlamydomonas genome sequencing was completed in 2007. Genomic G + C content is high and 64% (Merchant et al., 2007).

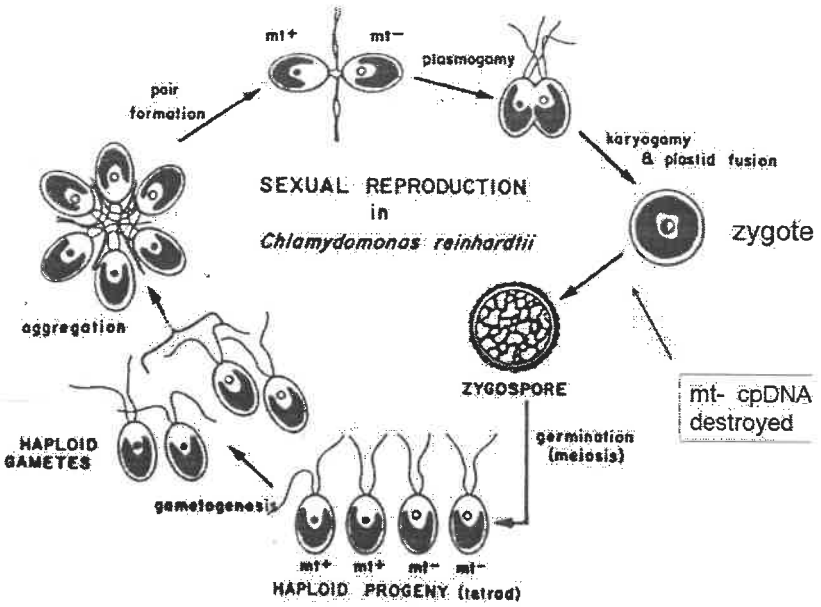

Figure 2.2. Life cycle of *C. reinhardtii* (Harris, 2009).

2.4. Genetic changing application to *Chlaydomonas reinhardtii*

There are several proven applications possibilities for genetic manipulation in *Chlamydomonas*. The target gene can be temporarily silenced by RNA interference (Schroda, 2006), or artificial miRNAs (Molnar et al., 2009; Hu et al., 2014), and CRISPR interference (Wang et al., 2014) could be used. Out of these, conditional transient silencing could be performed using inducible promoters by nitrate, nickel or CO_2. Thefore, the strains where all these methods are applied return to their original state after a few months, and because of this, they can not be used commercially.

Although homologous recombination experiments have been conducted to achieve permanent genetic modification, the extremely low homologous recombination rate in *Chlamydomonas* makes it difficult to implement alone (Sizova et al., 2013). Zinc finger nuclease application increases the efficacy ratio but its use is limited (Zorin et al., 2009).

Although transcription activator-like effectors (TALE) are applicable to target the desired DNA sequence, the fact that TALE-nuclease (TALEN) causes toxic effect on the cell allows TALE to function as an activator only in *Chlamydomonas* (Gao et al., 2014).

Finally, clustered regularly inter spaced palindromic region (CRISPR)-cas9 applications have been tested by researchers. Despite the demonstration of the success of the application, the transfer of the system component Cas9 as a vector to the cell has made it difficult to obtain permanent mutant strains due to cytotoxic effect after 24 hours (Jiang et al., 2014). Although the danger of toxicity has been overcome by transferring Cas9 as a purified protein to the cell, the fact that the mutation frequency is too low indicates that the application still needs to be developed (Jiang et al., 2014; Shin et al., 2016)

2.5. Carbon Concentration Mechanisms

Photosynthetic organisms have developed various strategies to adapt to adverse environmental conditions. The CO_2 concentration mechanism (CCM) is one of these. The task of CCM is to compensate for the low efficiency of ribulose-1.5-biphosphate carboxylase / oxygenase (Rubisco), the central enzyme for photosynthetic carbon assimilation, by increasing photosynthetic activity. Rubisco catalyses the first and most important reaction of the Calvin cycle by introducing CO_2 and ribulose-1.5-biphosphate into the reaction and is known to exhibit slow catalytic rate, low affinity to CO_2, and catalytic functions of carboxylases and oxygenases in photosynthetic organisms (Spreitzer and Salvucci, 2002). Existing atmospheric CO_2 concentration is well below the concentration required for Rubisco's carboxylase activity (Raven et al., 2008) and the high O_2/CO_2 ratio in the atmosphere increases the enzyme's oxygenase activity, resulting in energy expenditure and release of bound CO_2. In response to all these challenges, various strategies have been developed by the organisms to increase the CO_2 concentration around Rubisco and, at the same time, to achieve high photosynthetic performance by increasing the carboxylase activity. These strategies have been revealed in different species and range from C4 metabolic pathways in high plants to different forms of CCM in microalgae (Giordano et al., 2005; Sage et al., 2012).

2.6. Comparing C3 and C4 Photosytnthesis Systems

C4 plants such as corn, sugarcane and sorghum have about 50% more photosynthesis activity than C3 plants such as rice, wheat and potatoes. This is due to different carbon fixation mechanisms used in two different photosynthesis systems (Figure 2.3). C3 plants use Rubisco only in the Calvin cycle to bind CO_2, and this reaction takes place in chloroplasts of mesophyll cells. C4 plants perform photosynthetic reactions in mesophyll and bundle cells. The first carbon linkage takes place by the catalysis of the phosphoenolpyruvate carboxykinase (PEPC) enzyme, the formation of oxaloacetate (OAA) from CO_2 and phosphoenolpyruvate (PEP). OAA is transformed into malate and passes into the sheath cells of malt Rubisco, causing decarboxylation and increased CO_2 concentration around Rubisco. Finally, PEP, the starting substructure of C4 cycle, is regenerated by pyruvate orthophosphate dikinase (PPDK) (Kajala et al., 2011) (Figure 2.3).

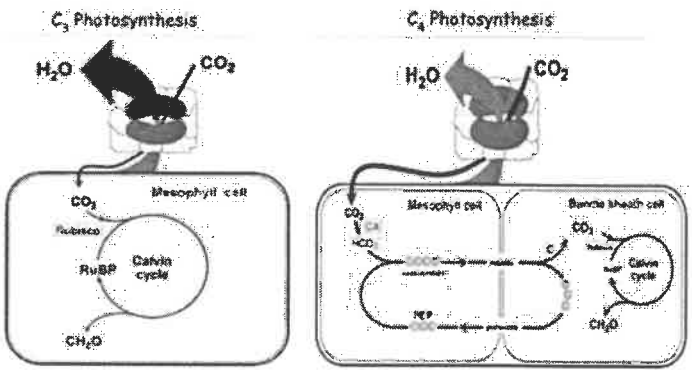

Figure 2.3. Schematic diagram of C3 and C4 photosynthesis systems (Wang et al., 2012).

2.7. Aquatic Photosynthetic Microorganisms

Aquatic photosynthetic microorganisms count for 50% of the world's photosynthesis. These organisms face some difficulties when CO_2 is taken from nature. The most important of these is that the diffusion of CO_2 from the liquid medium is 10,000 times slower than the diffusion from air.

Algae are thought to have developed C4-like photosynthesis systems against these difficulties, and these C4-like systems have been uncovered in the green macroalgae *Udotea flabellum* and the planktonic diatom *Thalassiosira weissflogii* (Reiskind et al., 1988 & 1991).

2.8. Pyruvate Orthophosphate(Pi) Dikinase(PPDK)

PPDK is one of the key enzymes of C4 photosynthesis, is considered a prime candidate (Sugiyama et al. 1979; Usami et al. 1995).PPDK is very important for PEP regeneration in mesophyll cells of NADP-ME and NAD-ME type species(Smith and Woolhouse, 1983). Two maize genes, *Pdk1* and *Pdk2*, produce for the isoforms PPDK1 and PPDK2, one by one, and *Pdk1*produces alternative transcripts that encode both a cytosolic and a plastidial form (Sheen,1991; Miyao, 2003).

PPDK was at the beginning characterized as a plastidial enzyme that produce the phospho*enol*pyruvate (PEP) that is used for CO_2 fixation in C4 plants (Hatch,1987). The enzyme changes pyruvate to PEP, using ATP and inorganic phosphate, also generates AMP and inorganic pyrophosphate (PP_i) as products. PPDK besides is present in nonphotosynthetic tissues, including endosperm from maize, wheat, and rice (Meyer et al., 1982; Aoyagi and Bassham, 1984; Sadimantara et al., 1996; Vensel et al.,2005; Mechin et al.,2007). In maize, the *Pdk1* gene encodes for both cytosolic and plastidial verisons of PPDK, however *Pdk2* has been thought to code for a cytosolic protein based on the absence of a recognizable transit peptide sequence (Sheen, 1991). But, PPDK2, found as an internal granule-associated protein (Boren et al., 2004), implying a plastidial location for at least a portion of the protein. One possible role of PPDK in endosperm, where C4 metabolism is not active, is the production of three carbon compounds used as precursors for amino acid biosynthesis (Chastain et al., 2006). Other role proposed recently is that PPDK serves as a regulator of carbon partitioning between starch and protein during grain filling in the endosperm (Mechin et al., 2007).

3. MATERIALS AND METHODS

3.1. Algal Strains and Culture Conditions

Chlamydomonas reinhardtii cells (strain CC-125, obtained from Chlamydomonas Genetics Center, Duke University) grown on Tris/acetate/phosphate (TAP) agar plates were inoculated in 500 mL Erlenmeyer flasks containing 100 ml TAP liquid medium. TAP medium consisted of NH_4Cl (7.48 mM), MgSO4 (406 mM), $CaCl_2$ (340 mM), K_2HPO_4 (540 mM), KH_2PO_4 (463 mM), 20 mM Tris, 17.4 mM acetate, H_3BO_3 (184mM), $ZnSO_4$ (76.5mM), $MnCl_2$ (25.5 mM), $FeSO_4$ (17.9 mM), $CoCl_2$ (6.77 mM), $(NH_4)6Mo_7O_{24}$ (0.88 mM), $CuSO_4$ (6.29 mM), and Na_2EDTA (148 mM). Cells were grown to late log phase, then were harvested by centrifugation and re-suspended at a starting density of 2×10^6 cells/mL in TAP media. All cultures were maintained at 25 °C and 100 µmol m^{-2} s^{-1} with continuous shaking at 200 rpm. After growth cultures were separated from the media by centrifugation at 5000 rpm for 15 min and the pellets were washed twice with distilled water to remove the salts. Then the pellets were ground in a mortar containing liquid N_2 and powders stored at -80 °C for later analysis.

Figure 3.1. Cultivation of *C. reinhardtii* cells for 4 days in the standard TAP-medium

3.2. RNA Isolation and Single Strand cDNA Synthesis

Total RNA was prepared using TRIzol Reagent (Ambion, USA). ~100 mg liquid N_2 frozen microalgae biomass was incubated in 1 ml TRIzol (Ambion, USA) at room temperature for 5 min. Then 0.2 mL chloroform was added into lysate and the tube was

shaken vigorously by hand for 15 s and incubated at room temperature for 2–3 min. The sample was centrifuged at 12,000.00 g for 15 min at 4°C. ~400 µL colorless upper phase containing the RNA was transferred to a fresh RNase-free tube. An equal volume of 70% ethanol was added to it to obtain a final ethanol concentration of 35% and vortexed. Total RNA was purified from the aqueous phase of TRIzol extract using the PureLink RNA Mini Kit (Invitrogen, USA) following manufacturer recommendations.RNA samples were pre-treated with 2 units of DNase I for 1 h at 37°C, after which the enzyme was inactivated at 65°C in the presence of 2 mM EDTA. This DNA-depleted RNA preparation was used for synthesis of the first-strand cDNA. The first-strand cDNA synthesis was performed by using Maxima First Strand cDNA Synthesis Kit (Thermo. Scientific, USA) with oligo (dT)18 primer according to manufacturer instructions. Samples of the first strand cDNA were used in PCR reactions to amplify full-length CrPPDK gene as described below.

3.3. Amplification of the C. *reinhardtii* CrPPDK gene

For PCR amplification of CrPPDK gene the following gene-specific primer-pairs were used: [5'-ATGGCTCCCATTCGCCGGATTTAC-3' (forward 1) and 5'-TTACTTGGCCTTAGCGGCCGC-3'(reverse1)] and [5'-ATGGCTCCCATTCGCCGG-3' (forward 2) and 5'-TTACTTGGCCTTAGCGGCCG-3' (reverse 2)]. PCR reaction was performed in a final volume of 50 µL containing 1x reaction buffer,1x high GC Enhancer, 1 µmol of each primer, 0.2 mM dNTPs, 1 M Betain and 0.01 U Q5 DNA polymerase (NEB, USA) using the following program: 98°C initial denaturation, 35 cycles of 98°C denaturation for 10 s, a 54-59°C annealing periods of 20 s and 72°C extension period of 1 min, and final extension at 72°C for 2 min, using 0.5-2.5 µL cDNA, prepared as described above, as template. PCR produced was analyzed in 1% agarose gel.

Table 3.1. Primers of *PPDK* gene for amplification and cloning

Primer	Primer sequences
PPDK F1	5'- ATGGCTCCCATTCGCCGGATTTAC -3'
PPDK R1	5'- TTACTTGGCCTTAGCGGCCGC -3'
PPDK F2	5'- ATGGCTCCCATTCGCCGG -3'
PPDK R2	5'- TTACTTGGCCTTAGCGGCCG -3'
pETPPDKF1	5'- GATCAC**CATATG**GCTCCCATTCGCCGGATTTAC -3'
pETPPDKR1	5'- ACTGTC**CTCGAG**TTACTTGGCCTTAGCGGCCGCC -3'

3.4. Construction and Extraction of Recombinant His6-tagged C. *reinhardtii* PPDK Protein

In order to construct the recombinant protein expression plasmid pET-28a(+)-PPDK, primer pairs were designed on the basis of the CrPPDK sequence. PCR was performed as described above using pBluescript- CrPPDK. The respective sequences of the forward and reverse primers used to construct pET-28a(+)-PPDK were 5'-GATCAC**CATATG**GCTCCCATTCGCCGGATTTAC-3' and 5'-ACTGTC**CTCGAG**TTACTTGGCCTTAGCGGCCGCC-3'. The bold underlined nucleotide bases indicate restriction-enzyme digestion-sites for *NdeI* and *XhoI*,

respectively. After amplification, the PCR product was purified with DNA Clean and Concentrator Kit (Zymo Research, USA). Concentrated PCR product (CrPPDK) and pET-28a(+) vector were digested by *NdeI* and *XhoI* for 10 min at 37°C, after which *NdeI* and *XhoI* were inactivated at 65°C for 5 min. Digested PCR pvector were loaded on a 1 % agarose gel for recovery and electrophoresed at 130 V for 1 h. Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) was used for next recovery processes following to manufacturer instructions. Then pET28a(+) vector was directionally ligated with CrPPDK gene. Ligation reaction was performed with 2 µL pET28a(+) vector, 1 µL CrPPDK gene, 7 µL water, 10 µL 2x Ligation Buffer and 1 µL Quick Ligase (NEB, USA) for 20 min at room temperature. Immediately the ligated plasmid was transformed into component cells of E. *coli* strain DH5α using the heat shock method. According to this method the ligation product was added into DH5α component cells, incubated on ice for 3-5 min to thaw the competent cells. Then the mixture was kept at 42°C water bath for 50 s and immediately was transferred to ice for 5 min to reduce damage to the E. coli cells. 400 µL LB Medium was added into the sample, incubated at 37°C, 225 rpm for 1 h. 50 µL of the resulting culture was spread on LB plate with 50 µg/mL kanamycin, incubated at 37°C overnight. About 16 h later colonies were selected from the plate, inoculated into 3 mL LB Medium with 50 µg/mL and incubated 37°C overnight. The plasmid from DH5α was purified using Zyppy Plasmid Miniprep Kit (Zymo Resaearch, USA) according to manufacturer instructions. Purified plasmid from DH5a was re-transformed into E. coli strain BL21(DE3) competent cells (Novagen) to express recombinant CrPPDK protein. Cultures of E. coli strain BL21(DE3), transformed with pET-28a(+)-PPDK, were grown at 37°C in 200 mL of LB medium containing 50 µg/mL kanamycin to an OD_{600} of ~0.6. Recombinant protein was induced with or without 1 mM isopropyl-b-D-thiogalactoside (IPTG) in order to express CrPPDK for 3 h at 37°C. Subsequently, the cells were harvested at 4°C by centrifugation at 5000 g for 15 min and were ground in a mortar containing liquid N_2. The powder was re-suspended in 10 volumes of ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM DDT, 10 mM MgSO4, 1 mM EDTA, 2 mM KH2PO4, 10 mM 2-mercaptoethanol, centrifuged at 15 000 rpm for 15 min.

3.5. SDS-PAGE and İmmunoblotting

For immunoblot analysis, SDS-PAGE was performed on 10 % acrylamide gels, and the gels were subsequently blotted to polyvinylidene difluoride membranes using a 25 mM Tris base/150 mM Gly, pH 8.3, transfer buffer at 100 V for 1 h. The membranes were first blocked with 5% (w/v) low-fat milk in TBS (Tris Buffered Saline) at room temperature for 1 h, and then probed with the His6·Tag monoclonal antibody (Novagen, USA) in TBS containing 5 % (w/v) low-fat milk for 1 h at room temperature. After three 10-min washes, the membranes were incubated with a goat anti-mouse IgG H&L (HRP) (Abcam, USA) for 1 h at room temperature, followed by five 10-min washes with TBS, and finally chemiluminescent detection was performed with the ECL western-blotting reagents from Amersham Biosciences). Images were taken using high sensitive GeneGnome XRQ Chemiluminescence imaging system (Syngene, A Division of Synoptics Ltd).

3.6. PPDK Enzym Activity Assay

Samples of 150-200 mg liquid N_2-frozen C. *reinhardtii* or E. *coli* cells were homogenized in ice-cold extraction medium. The PPDK extraction medium contained 50 mM Tris-HCl (pH 7.4), 10 mM DDT, 10 mM $MgSO_4$, 1 mM EDTA, 2 mM KH_2PO_4, 10 mM 2-mercaptoethanol. Enzyme activities were assayed spectrophotometrically by following changes in A340 at 25°C. The assay mixture for PPDK contained 25 mM Hepes-KOH, pH 7.4, 6 mM $MgSO_4$, 10 mM DDT, 25 mM NH_4Cl, 1 mM PEP, 0.5 mM AMP, 1 mM PPi, 0.25 mM NADH and 2 U/ml of LDH. One international unit (U) of enzyme activity is defined as the amount of enzyme resulting in the production of 1 µmol of product per min at 25°C. Soluble protein concentration was determined using the Bradford Protein Assay Reagent.

3.7. PPDK Activities in *C. Reinhardtii* Under Different Growth Conditions

PPDK enzyme activity was measured in C. *reinhardtii* cells grown at high and low CO_2 and NH_4^+ ratios. Cells were grouped as 2-5% CO_2 and grown in air and grown in 7.48, 1.87 and 0 mM NH_4^+, and these groups were compared within themselves.

4. RESULTS AND DISCUSSION

4.1. Amplification and Cloning of *C. Reinhardtii PPDK* Gene

The recent released C. *reinhardtii* genomic data provided evidence for existence of the PPDK gene in the Chlamydomonas data base. The nucleotide sequence information (mRNA sequences) for PPDK gene also available in Chlamydomonas GenBank under accession numbers, XM_001702520.1. Based on this sequence, we designed primers to amplify full length of the PPDK genes from C. reinhardtii cDNA. For PCR amplification of CrPPDK gene the following gene-specific primer-pairs were used: [5'-ATGGCTCCCATTCGCCGGATTTAC-3' (forward1) and 5'-TTACTTGGCCTTAGCGGCCGC-3' (reverse 1)] as described in Materials and Methods. PCR reaction were performed in a final volume of 50 µl containing 1× PCR reaction buffer, 1X GC Enhancer 0.5 µM of each primer, 0.2 mM dNTPs, and 0.02 U/µl Q5 DNA polymerase (NEB, USA) using the following program: 98°C initial denaturation for 30 sec, 35 cycles of 98 °C denaturation for 10 sec, a 56 °C annealing period of 20 sec and 72 °C extension period of 1:30 min, and final extension at 72°C for 2 min, using 2 µl cDNA library, as template. PCR amplication of CrPPDK gene was shown in Figure 4.1. As can be seen from Figure 4.1, an expected size (2676 bp) PCR product was amplified.

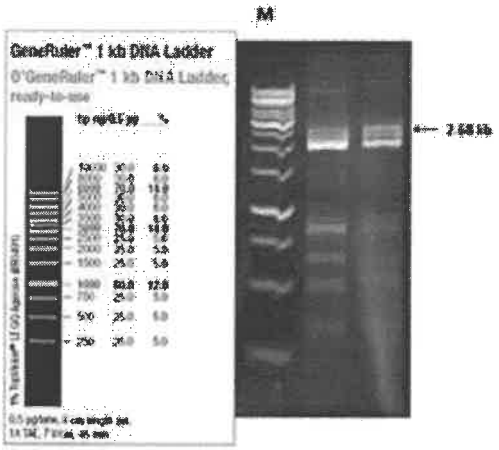

Figure 4.1. PCR application of CrPPDK gene from cDNA *C. reinhardtii*. Amplification of CrPPDK gene was shown as indicated. M:GeneRuler 1kb DNA Ladder (Thermo Scientific, in the left) used as DNA ladder. *CrPPDK* ORFs were further cloned into pBluescript II vector (Stratagene), previously digestion with EcoRV.

4.2. Construction of pET-28a(+)-*PPDK* plasmid expression recombinant *PPDK* protein

In order to verify experimentally that *CrPPDK* gene encode functional enzyme, the corresponding ORF was subcloned into the *E. coli* expression vector pET-28a (+) and transformed into expression strain BL21(DE3). In order to construct the recombinant protein expression plasmid pET-28a(+)-PPDK, primer pairs were designed on the basis of the *CrPPDK* sequence. PCR was performed as described in Materials and Methods using pBluescript-CrPPDK. The respective sequences of the forward and reverse primers

used to construct pET-28a (+)-PPDK were 5'-GATCAC**CATATG**GCTCCCATTCGCCGGATTTAC-3' and 5'-ACTGTC**CTCGAG**TTACTTGGCCTTAGCGGCCGCC-3'. The bold underlined nucleotide bases indicate restriction-enzyme digestion-sites for *NdeI and XhoI*, respectively. After amplification, the PCR product was purified with DNA Clean and Concentrator Kit (Zymo Research, USA). Concentrated PCR product (*CrPPDK*) and pET-28a(+) vector were digested by FD *NdeI* and FD *XhoI* for 10 min at 37 °C, and then *NdeI* and *XhoI* activites were inactivated at 65°C for 5 min. Digested PCR product and pET-28a(+) vector were loaded on a 1 % agarose gel for recovery from the gel. Cloning of the *CrPPDK* fragment into pET-28a (+) plasmid, expression in *E. coli* BL21(DE3) competent cells (Novagen) were performed as described in Materials and Methods. As a result, recombinant *CrPPDK* was expressed as a fusion protein with a 20-residue N-terminal extension. The expression plasmids were re-isolated and digested with *NdeI-XhoI* (Figure 4.3B) in order to verify that the ligations and the CrPPDK insert was correctly in-frame. As can be seen from Figure 4.3B, after digestion with *NdeI and XhoI* restriction enzymes, right size vector and insert were observed in agarose gel.

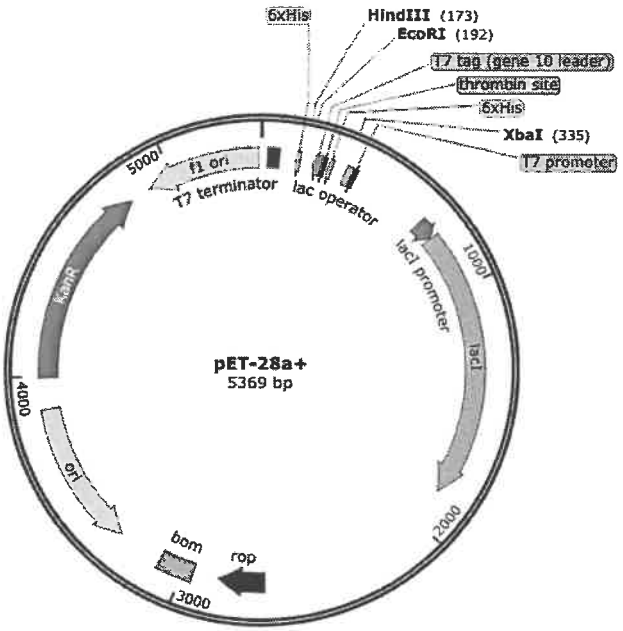

Figure 4.2. Map of pET-28a(+) plazmid vector(Snapgene).

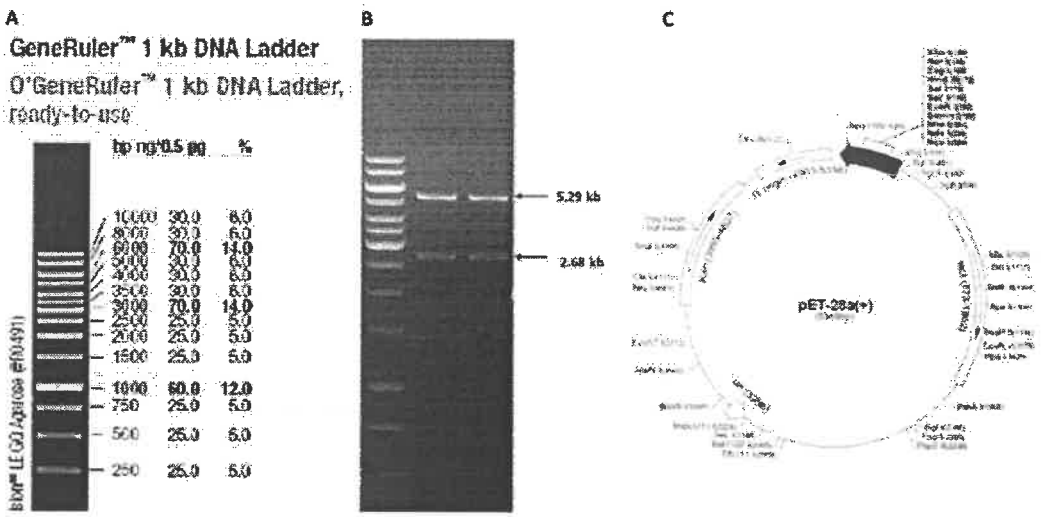

Figure 4.3. Digestion of pET-28(a)-Cr*PPDK* with *NdeI and XhoI* rescriction enzymes. A: *GeneRuler 1kb DNA Ladder (*Thermo Scientific, in the left) used as DNA ladder in Figure 4.3 B. B: pET-28(a) – CrPPDK digested with *NdeI and Xho I* restriction enzymes. C: map of plasmid pET-28(+) vector.

4.3. Extraction of recombinant, His6-tagged *C. reinhardtii* PPDK protein

Cultures of *E. coli* strain BL21(DE3), transformed with pET-28a(+)-PPDK, were grown at 37°C in 200 ml of LB medium containing 50 µg/ml kanamycin to an OD_{600} of ~0,6. Recombinant protein was induced with or without 1 mM isopropyl-b-D-thiogalactoside (IPTG) in order to express *rCrPPDK* for 3 h at 37°C. Subsequently, the cells were harvested at 4°C by centrifugation at 5000 *g* for 15 min and were ground in a mortar containing liquid N_2. The powder was re-suspended in 10 volumes of ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM DDT, 10 mM MgSO4, 1 mM EDTA, 2 mM KH2PO4, 10 mM 2-mercaptoethanol and stored at −20°C until used.

The expressed recombinant PPDK protein was found to be highly soluble and was evidenced by western blot analysis (Figure 4.4). As can be seen from figure, recombinant PPDK protein migrates in a gel as ~95 kDa protein.

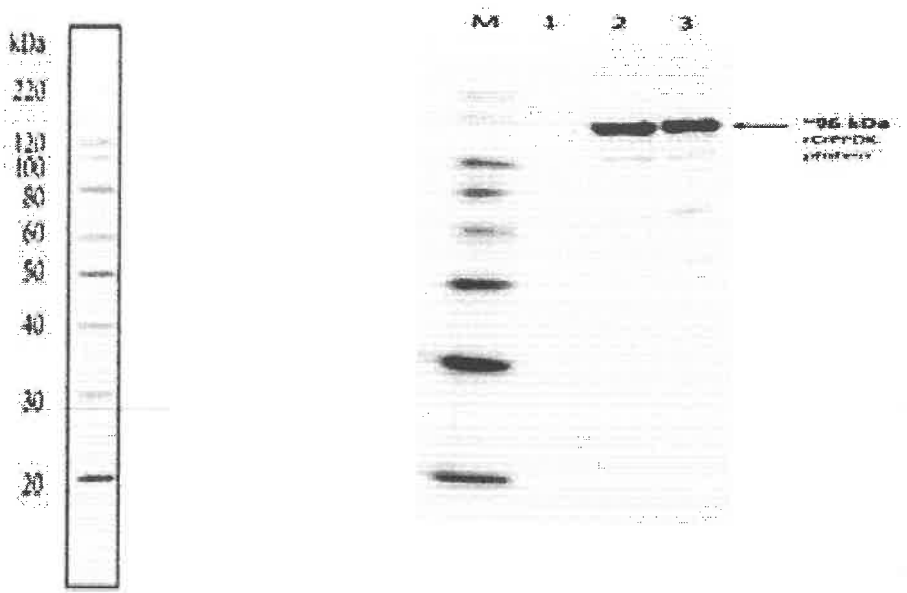

Figure 4.4. Immunoblot analysis of CrPPDK recombinant protein expressed and extracted from *E. coli* strain BL21(DE3). A: 1 – Protein extract of *non-transformed* E. coli (control), 2 – Protein extract of transformated *E. coli* with no IPTG added; 3: Protein extract of transformated *E. coli* with IPTG added M – Marker.

When assayed at optimal conditions, the specific activity of PPDK in transformed cell extracts was 3,12 fold higher compare in the extracts of control cells. These results indicate that the *C.reinhardtii* recombinant PPDK produced in *E. coli* was fully active and that their modest, 20 residue N-terminal extensions had no detrimental effect on activity. Thus, these collective data demonstrate that *CrPPDK* gene in functionally and enzymatically active in *C.reinhardtii* cells and can be candidate for knock out the gene for possible oil increase in *C. reinhardtii* cells.

4.4. PPDK activities in *C. reinhardtii* grown cells under different NH_4Cl concentrations

We developed procedure to determine PPDK activities in *C. reinhardtii* grown under different NH_4Cl concentrations. The results were presented in Table 4.1, although the specific activity of PPDK vary slightly, but the total activity of PPDK was significantly (about 3 times) increases with NH_4Cl concentration in the TAP-medium and was less total enzymatic activity in the absence of NH_4C in growth medium, which is associated with a high oil content (see below table 4.1). Indeed, *C. reinhardtii* cells grown in the absence of NH_4C had comparable high oil content. This data indicate that the knockdown of gene *CrPPDK* can increase the oil content in the *C. reinhardtii* cells.

Table 4.1. PPDK activities of *C. reinhardtii* cells grown under different NH_4Cl concentrations in TAP-medium

NH_4Cl concentration in TAP-medium, mM	Protein concentration in extract, mg/ml	Total enzyme activity, U/ml $\times 10^{-3}$	Enzyme activity, U/mg soluble protein $\times 10^{-3}$	Enzyme activity, U/g fresh weight $\times 10^{-3}$
7.48	9.46	127.8	13.5	384
1.87	4.84	72.9	15.1	219
0.00	3.10	48.1	15.5	144

Table 4.2. PPDK activity assay in extracts of *E.coli*, non-trasformed and transformed with CrPPDK gene

E. coli	Protein concentration in extract, mg/ml	Total PPDK activity, U/ml $\times 10^{-3}$	PPDK activity, U/mg soluble protein $\times 10^{-3}$
Non-transformed	1.56	44.5	28.5
Transformed	0.50	44.5	89

4.5. Activity and gene expression analysis of PPDK of *C. reinhardtii* cells grown in high CO_2 and switched to air or grown in ambient air and then switched to air.

Previously above we were showed about cloning and heterelogous expression of PPDK gene from *C. reinhardtii* for the first time. We also tested on activity analysis of PPDK enzyme at different growth condition of *C. reinhardtii*. In this process, we performed expression and activity analysis of PPDK of *C.reinhardtii* cells, grown in high CO_2 (5%) or ambient air, and then switched to air or high CO_2 (5%), respectively. Expression analyses was performed by qPCR as described in Materials and Methods. Results are presented in Figure 4.5.

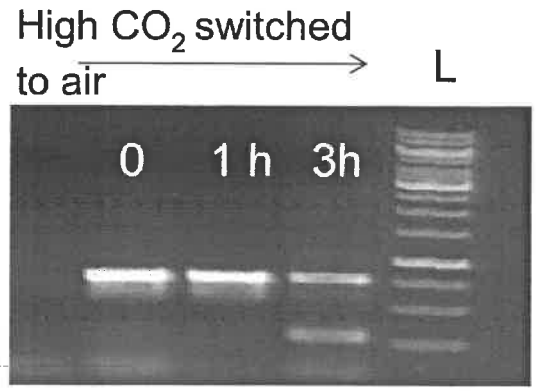

Figure 4.5 Expression analysis of analysis of PPDK genes in *C. reinhardtii* cells grown in high/low levels of CO_2.

As can be seen from Figure 4.3, PPDK gene is CO_2-responsive gene and its steady-state transcript levels are up-/down-regulated by varying levels of CO_2 supplied to the growth medium. Activity of PPDK of *C. reinhardtii* cells grown in high CO_2 and switched to air (0.5, 1.0 and 3.0 hours) or grown in ambient air and then switched to air (0.5, 1.0 and 3.0 hours) have been presented in Table 4.3. Enzymatic activity analysis showed that activate of PPDK enzyme is correlated with gene expression. Thus, these results show that a change in the level of this gene may lead to an increase in the oil level in *C. reinhardtii* cells.

Table 4.3. Activity of PPDK of *C. reinhardtii* cells grown in high CO_2 and switched to air (0.5, 1.0 and 3.0 hours) or grown in ambient air and then switched to air (0.5, 1.0 and 3.0 hours).

	Protein, mg/ml	Total activity, U	Specific activity, U/mg	Specific activity, %	Fold
High CO_2 grown cells	1.40	27.28	19.48	100	1
High CO_2 switched to air 0.5 h	1.302	3.486	2.68	13.49	7.41 Fold decrease
High CO_2 switched to air 1.0 h	0.779	3.422	4.39	22.14	4.52 Fold decrease
High CO_2 switched to air 3 h	0.885	4.610	5.21	26.73	3.74 Fold decrease
Air grown cells	1.325	4.243	3.20	100	1
Air grown cells switched to High CO_2, 0.5 h	1.180	40.69	3.44	107.43	1.07 Fold increase
Air grown cells switched to High CO_2, 1.0 h	1.063	13.20	13.16	410.99	4.11 Fold increase
Air grown cells switched to High CO_2, 3h	1.123	14.01	12.47	389.57	3.90 Fold increase

4.6. Total neutral lipid level determination development

We developed a method to determine neutral lipid levels in *C.reinhardtii* cells as described Materials and Methods. As shown in table 4.4, *C. reinhardtii* cells growing in the absence of NH_4Cl in TAP-medium had a high content of total neutral lipids (Table 4.4).

Table 4.4 Relative dry weight and total neutral lipid levels (on a dry weight basis) in *C. reinhardtii* under different growth conditions (different NH_4Cl concentrations in TAP-medium).

NH_4Cl concentration in TAP-medium, mM	Dry weight (%)	Total neutral lipid (%)
7.48	13.1	8.7
1.87	11.1	14.4
0.00	9.6	22.5

5. CONCLUSIONS

Microalgae are a wide variety of microorganisms and can produce large amounts of TAG under photo-oxidative stress or other adverse environmental conditions. TAG synthesis pathways have not yet been elucidated in algae using molecular biology methods. TAG biosynthetic pathways are central to the storage of carbon and energy in cellular stress situations. Thanks to the photosynthetic activity and growth potential of the algae, it is possible to obtain about 200 barrels of algal oil on a daily basis from 1 hectare area. However, due to cost increases in the isolation and conversion of this oil, the production of algal oil needs to be further increased.

Our long-term research goals are engineering of *C. reinhardtii* metabolism to enhance the accumulation of triacylglycerols (TAGs) while maintaining efficiency of carbon dioxide conversion. The immediate goal of this project is detailed molecular and biochemical characterization of pyruvate, orthophosphate (Pi) dikinase (PPDK) gene for further knock out of these genes in *C. reinhardtii*. Our central hypothesis is that a detailed characterization of C4 methabolisim in *C. reinhardtii* may lead to strategies for increasing the levels of triacylglycerols (TAGs), by modifying levels of storage oil/protein, keeping in mind that i) photosynthetic carbon flux tends to synthesize proteins or lipids depending on the relative activity and ii) lipids and proteins of cells compete for the same substrate, i.e., pyruvate, a product of glycolysis, and iii) the levels of pyruvate and TAG can be significantly altered/regulated by C4-methabolism enzymes.

Green algae C. *reinhardtii* is a useful model for photosynthetic organisms and has been used for many physiological process investigations. Recently, several molecular technologies, such as nuclei, organelle transformation, RNAi, have been shown to be viable in Chlamydomonas. These properties make *Chlamydomonas* an idelal algal organism for the investigation of pathways associated with TAG biosynthesis.

C4 photosynthetic metabolic pathways are essential for the attachment of at high rates carbon dioxide to plants. The search for photosynthesis systems is important not only for crop production but also for the production of plants with high oil content. What happened was that the C4 pathway enzymes were not investigated in the algae at molecular level enough. The investigation of PEPC genes in C. reinhardtii is the first work to be done in this area (Mamedov et al 2005). Because of the inadequate work in this area, C4 metabolism is very important in the C. reinhardtii of the model organism genes. PEPC, PPDK and PEPCK are the main enzymes of the C4 cycle. PEPCK is thought to negatively affect TAG storage by introducing the enzyme into the pyrolytic reaction, which is important for TAG synthesis in algae. It is anticipated that the TAG ratio in the algae will increase as the activity of the PEPCK enzyme decreases.

In this study, it was shown that PPDK was highly active and by cloning and enzyme activation measurement in E. coli it has shown. In addition, western blot and SDS-PAGE methods also revealed the expected protein sizes and the . Changes in PPDK in different NH_4+ and CO_2 ratios in C.*reinhardtii* have shown that the enzyme may be photosynthetic and negative in relation to fat gain. Furthermore, as seen in the PCR

results, the decrease in expression by decreasing the CO2 concentration in the medium suggests that this gene is also related to the carbon flow in the organism.

As a result of all these studies, it has been understood that the isolation of PPDK gene alone or in combination with other photosynthetic enzyme genes in algae could increase the fat ratio to a large extent and produce an industrially important algal strain.

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