Molecular and Biochemical Studies of Astaxanthin Biosynthesis in *Haematococcus pluvialis*

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BIOTECHNOLOGY

By

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Dedicated.....

To My Family,Friends & Guide

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DECLARATION

I, **R. Vidhyavathi**, certify that this thesis entitled "**Molecular and biochemical studies of astaxanthin biosynthesis in *Haematococcus pluvialis*"** is the result of research work done by me under the supervision of **Dr. R. Sarada** at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore- 570 020, India during the period 2003 - 2008. I am submitting this thesis for possible award of **Doctor of Philosophy (Ph.D.)** degree in **BIOTECHNOLOGY** of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree / diploma of this or any other University.

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CERTIFICATE

This is to certify that the thesis entitled "**Molecular and biochemical studies of astaxanthin biosynthesis in *Haematococcus pluvialis*"** submitted by **Ms. R. Vidhyavathi** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is the result of research work carried out by her under my guidance in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** during the period September 2003 to December 2008.

Place: Mysore
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R. Sarada
(Research Supervisor)

Abstract

Haematococcus pluvialis is a commercially promising source of astaxanthin **(**3,3'-dihydroxy-β,β-carotene-4,4'-dione) which is mainly used as a pigmentation source in the aquaculture and poultry industries. The present work is focused on molecular and biochemical studies of astaxanthin biosynthesis in *H. pluvialis*. Nutrient stress (modified autotrophic medium containing $1/10^{th}$ of N and P) and higher light intensity (60 μmol m^{-2} s^{-1}) in combination with 17.1 mM NaCl / 4.4 mM sodium acetate enhanced total carotenoid and total astaxanthin content to 32.0 and 24.5 mg g^{-1} of dry biomass, respectively. Expression of carotenoid biosynthetic genes revealed that they are up-regulated and maximum transcript levels of phytoene synthase, phytoene desaturase, lycopene cyclase, β-carotene ketolase and β-carotene hydroxylase genes were found to be 158–277, 5–9, 470–674, 28–40, and 451–673-fold higher, respectively, than that in green vegetative cells under stress conditions. The maximum content of astaxanthin recorded in cells grown in medium with sodium acetate and NaCl/sodium acetate correlated with the expression profile of the astaxanthin biosynthetic genes. Both general carotenogenesis and secondary carotenoid induction were regulated at transcriptional and cytoplasmic translational levels. This study also suggested a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes. Experiments using inhibitors of carotenoid and fatty acid synthesis indicated the involvement of other regulatory factors besides transcriptional regulation of carotenogenesis in *H. pluvialis*.

Studies using photosynthetic inhibitors showed that the expression of photosynthetic genes, *cabL1818*, *lhcbm9*, *psaB* and *rbcL* were under redox control of plastoquinone pool and *atpB* gene expression may be regulated at cytochrome *b6/f* complex. The nuclear genes *cabL1818* and *lhcbm9* are coding for the chlorophyll *a/b* binding protein L1818 and major light-harvesting complex II m9 protein respectively. The chloroplast encoded *psaB* gene codes for PSI reaction centre protein PsaB. The chloroplast genes *rbcL* and *atpB* are coding for large subunit of Ribulose bisphosphate carboxylase oxygenase (Rubisco) and ATP synthase β-subunit respectively. Expression of all five photosynthetic genes studied was regulated at transcriptional and cytoplasmic translational levels, and their expressions were reduced by norflurazon induced photo oxidative stress. Acetate modulates the high light induced

expression of photosynthetic genes and it depends on redox state of cytochrome *b6/f* complex and cytoplasmic protein synthesis.

The genes differentially expressed under the stress conditions were analysed by mRNA differential display RT PCR and 34 differentially expressed transcripts have been identified. These transcripts are having homology to molecules related to general metabolism, photosynthesis, carotenoid synthesis, lipid synthesis, tetrapyrrole synthesis, transporter proteins, defense signaling, genetic information processing and unknown function or shared no apparent homology to any expressed sequences in the GenBank/EMBL databases. A partial transcript homologue to *psaB* gene coding D1 protein of photosystem II has been identified for the first time in *H. pluvialis*. LCY and BKT activities were found to increase under stress condition. Maximum activity of BKT was observed in lipid globules of stress induced cultures. Pigment composition of cell fractions revealed that chloroplast fraction is having lutein, β-carotene and chlorophyll as pigments while lipid fraction is having β-carotene, astaxanthin, canthaxanthin and echinenone as pigments. Under the influence of HL, exposure of cells to nutrient deficiency enhanced carotenoid accumulation which was further enhanced by exposure to CO_2 enriched environment and/or NaCl and sodium acetate addition. Changing the carbonate buffer in the lower compartment of 2-tier vessel to provide constant CO_2 environment was found to be efficient in enhancing carotenoid content. Influence of phytohormones such as salicylic acid and methyl jasmonate on pigment production and antioxidant revealed that at lower concentrations these phytohormones could be used for elicitation of secondary carotenoid production.

Influence of few cultural parameters and temperature treatments on regeneration efficiency of red cysts along with changes in pigment profile and expression of carotenogenic genes during regeneration were investigated. Regeneration efficiency has been improved by incubating less aged cyst cells in medium containing ammonium carbonate, 16:8 light dark cycles with light intensity of 30 μmol m^{-2} s^{-1}. During regeneration there was decrease in total astaxanthin, total carotenoids and carotenoid to chlorophyll ratio, and increase in β-carotene, lutein, total chlorophyll and chlorophyll a to b ratio. Expression analysis of carotenogenic genes during regeneration of *H. pluvialis* cysts showed these transcripts were transiently up-regulated upon transfer to favorable conditions and later reached basal

expression levels of green motile vegetative cells. In addition, this is the first report of detection of carotenogenic gene transcripts in red cysts and their differential expression during regeneration. It is evident from the results that acetate has a role in regulation of both carotenogenic and photosynthetic gene expression. The results from the present studies will be helpful in understanding the regulation of carotenogenesis and metabolic engineering of carotenoid pathway.

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(R.Vidhyavathi)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BKT	:	β-carotene ketolase
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pairs
cDNA	:	Complementary Deoxyribonucleic Acid
Chl	:	chlorophyll
CHY	:	β-carotene hydroxylase
cm	:	Centimeter
d	:	day
DBMIB	:	2,5-dibromo-3-methyl-6-isopropyl-benzoquinone
DCMU	:	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DDRT-PCR	:	Differrential Display Reverse Transcription- PCR
DEPC	:	Diethyl-pyrocarbonate
DIG	:	Digoxigenin
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic Acid
DNase I	:	Deoxyribnuclease I
dsDNA	:	Double strand Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide triphosphate
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
EST	:	Expressed Sequence Tag
F0, Fv, Fm	:	minimum, variable and maximum chlorophyll fluorescence in dark-adapted state
Fv/Fm	:	maximum photochemical quantum yield of PSII
g	:	gram
h	:	hour
HL	:	high light
HPLC	:	High Performance Liquid Chromatography
IPTG	:	Isopropyl-β–D–thiogalactopyranoside
kb	:	Kilobase
kDa	:	Kilodalton
L	:	Litre
LB	:	Luria- Bertani (medium)
LCY	:	lycopene cyclase
LL	:	low light
M	:	Molar
mg	:	Milli gram
min	:	minute
ml	:	Millilitre
mM	:	Millimolar
MOPS	:	4-Morpholinepropanesulfonic acid
mRNA	:	messenger RNA
NBT	:	Nitroblue tetrazolium
NCBI	:	National Centre for Biotechnology Information
ng	:	nanogram
NF	:	norflurazon: [4-chloro-5-(methylamino)-2-(α, α, α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone]

nM	:	nanomolar
nt	:	nucleotide
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
PDS	:	phytoene desaturase
PET	:	photosynthetic electron transport
pmol	:	Picomol
PQ	:	plastoquinone pool
PS	:	photosystem
PSY	:	phytoene synthase
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	ribosomal RNA
ROS	:	Reactive Oxygen Species
RT	:	Reverse transcription
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
SA	:	Sodium acetate
SC	:	secondary carotenoid
ScA	:	Salicylic acid
SD	:	Standard Deviation
SE	:	Standard Error
SDS	:	Sodium dodecyl sulphate
SSC	:	Saline sodium citrate
TAE	:	Tris-acetate-EDTA
Taq	:	*Thermus aquaticus*
TBE	:	Tris-Borate-EDTA
TE	:	Tris-EDTA buffer
Tris	:	Tris (hydroxymethyl) amino methane
TLC	:	Thin Layer Chromatography
U	:	Unit enzyme
UV	:	Ultra Violet
V	:	Volt
v/v	:	Volume per volume
W	:	Weight
w/v	:	Weight per volume
X-GAL	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	:	Alpha
β	:	Beta
µg	:	Micro gram
µM	:	Micro molar
µl	:	Micro litre
%	:	Percent
ºC	:	Degree Centigrade
3’	:	Hydroxyl- terminus of DNA molecule
5’	:	Phosphate-terminus of DNA molecule

Introduction

G1. Introduction

Pigments are compounds that absorb light in the wavelength range of the visible region. They are widely distributed in living organisms and a large number of structures have been reported. Chlorophylls and carotenoids are the most abundant pigments in nature. They are involved in fundamental processes, and life on earth depends on them. Carotenoids provide many fruits and flowers with distinct red, orange and yellow colors and a number of carotenoid-derived aroma, and they are ubiquitous component of all photosynthetic organisms as they are required for assembly and function of the photosynthetic apparatus. In plants carotenoids participate in the light harvesting process as accessory pigments and protect the photosynthetic apparatus from photo-oxidative damage besides acting as membrane stabilizers. Animals do not synthesize carotenoids *de novo* and get it through diet. In animals, besides being as precursors for vitamin A and retinoids, carotenoids act as quenchers of singlet oxygen, free radical scavengers and antioxidants. They also play a protective role against some cancers, macular degenerative diseases etc.

Microalgal species are being exploited for large scale production of carotenoids. They produce wide spectrum of carotenoids viz., lutein, zeaxanthin, astaxanthin, canthaxanthin and β-carotene. Among the commercially important carotenoids, astaxanthin is used as a pigmentation source in the aquaculture and poultry industries and has found applications in the nutraceutical, pharmaceutical, and cosmetic industries. *Haematococcus pluvialis* is a commercially promising source of astaxanthin. It accumulates astaxanthin in extra plastidic lipid vesicles in response to stress conditions. In the past most of the work on *H. pluvialis* are related to cultural parameters, and morphological and physiological changes associated with carotenogenesis. With this background, *H. pluvialis* was selected as model organism to study the expression and regulation of carotenogenic genes.

G2. Scope and objectives of the present investigation

Secondary carotenoids are accumulated by very few organisms mainly to protect from photo-inhibition. The two distinct phases of life cycle, green motile vegetative phase and astaxanthin accumulating cyst phase, each with distinct carotenoid profile favors the use of *H. pluvialis* as a model system to study the regulation of carotenogenesis. Carotenoid biosynthetic genes of *H. pluvialis* are transcriptionally up-regulated and

are photosynthetic redox state regulated. Expression of carotenogenic genes has been studied in several plants including green algae, and transcription of carotenogenic genes were shown to be up-regulated by light (Bohne and Linden 2002; Simkin et al. 2003; Steinbrenner and Linden 2003; Romer and Fraser 2005) or a combination of light with N-deprivation (Grünewald et al. 2000). Although there are reports available on the influence of light on the expression of carotenogenic genes in *Haematococcus*, studies on the influences of other stress factors which are important for enhancing astaxanthin production on the expression of carotenogenic genes are limited (Grünewald et al. 2000; Huang et al. 2006a). Likewise, proteome and transcriptome studies to understand the molecular processes associated with carotenogenesis are mainly focused on effect of combination of stresses (Eom et al. 2006; Wang et al. 2004a, 2004b). Reports on molecular basis of carotenogenesis are limited in *H. pluvialis*. In addition, *H. pluvialis* cyst cells have remarkable ability to germinate or regenerate and produce large number of flagellated cells. Studies on this regeneration process of *H. pluvialis* cysts have been focused mainly on nutrient availability and interaction with light. There are no reports on regulation of carotenogenesis during regeneration. Understanding the molecular basis of stress-induced astaxanthin accumulation in *H. pluvialis* will be useful for the optimization of astaxanthin production. With this background knowledge, the objectives of the present research work were laid as follows:

1. To study the differential expression of the enzymes involved in astaxanthin biosynthesis in *Haematococcus pluvialis*
2. To study the biochemical changes associated with carotenogenesis

The results of the research work done systematically on the above objectives are compiled in the thesis, under the following chapters,

Chapter I: Review of literature

Chapter II: Expression of carotenoid biosynthetic genes and carotenoid accumulation

Chapter III: Regulation of carotenogenic and photosynthetic genes expression

Chapter IV: Molecular and biochemical changes associated with carotenogenesis

Chapter V: Expression of carotenogenic genes and associated changes in pigment profile during regeneration of *Haematococcus pluvialis* cysts

Chapter VI: Summary and Conclusions

Chapter I

Review of Literature

1.0. Carotenoids

Carotenoids are the most widespread group of pigments found in nature. They provide many fruits and flowers with distinct red, orange and yellow colors and a number of carotenoid-derived aroma, making them commercially important in agriculture, food manufacturing and cosmetic industries (Armstrong 1994; Ben-Amotz and Fishler 1998; Cuttriss and Pogson 2004). Carotenoids impart pigmentation to organisms as diverse as fish, crustaceans and birds. However, it is their role in photosynthesis and nutrition that make them essential for the survival of plants, animals and mammals alike. Specifically, carotenoids are ubiquitous component of all photosynthetic organisms as they are required for assembly and function of the photosynthetic apparatus. Carotenoids are also a vital part of our diet as antioxidants and precursors to vitamim A (Goodwin 1980; Cuttriss and Pogson 2004). Their distinctive colors, typically in the yellow to red spectrum, are due to a series of conjugated double bonds. The range of colors is expanded by diverse modifications to the simple polyene chain structure. Interactions of certain carotenoids with apoproteins, such as astaxanthin with the crustacyanin protein from shellfish, can result in blue pigmentation that shifts back to red during cooking as the proteins are denatured (Cuttriss and Pogson 2004). Although commonly thought of as plant pigments, carotenoids are also encountered in some animal foods. Animals are incapable of carotenoid biosynthesis, thus their carotenoids are diet derived, selectively or unselectively absorbed, and accumulated unchanged or modified slightly into typical animal carotenoids (Rodriguez-Amaya 2001).

So far, over 600 carotenoids have been characterized, of which about 50 are provitamin-A, which includes β-carotene, α-carotene, γ-carotene and β-cryptoxanthin (Pfander 1987; Straub 1987; Faure et al. 1999). Carotenoids are a large family of lipophilic isoprenoids. They are usually C_{40} tetraterpenoids built from eight C_5 isoprenoid units, joined by the head-to-head linkages so that the sequence is reversed at the center (Cunningham and Gantt 1998). The basic linear and symmetrical skeleton, which can be cyclized at one or both ends, has lateral methyl groups separated by six C atoms at the center and five C atoms elsewhere. Cyclization and other modifications, such as hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, introduction of oxygen

functions, or combinations of these processes, result in a myriad of structures. A distinctive characteristic of carotenoid is an extensive conjugated double-bond system, which serves as the light-absorbing chromophore responsible for the yellow, orange, or red color that these compounds impart to many foods (Sapozhnikov 1967; Cunningham and Gantt 1998; Rodriguez-Amaya 2001). Lycopene shows the acyclic hydrocarbon chain. This compound is regarded as the prototype of the family (Weeden and Moss 1995). Structural modifications of lycopene lead to the diverse nature of the carotenoids present in the plant kingdom. β-carotene is the most significant of the provitamin A carotenoids, characterized by the cyclicized β-ionone rings on both ends of the hydrocarbon chain. Carotenoids are synthesized as hydrocarbon carotenoids (i.e., carotenoids made up of only carbon and hydrogen and are collectively called as carotenes; e.g., lycopene, α-carotene, and β-carotene) or their oxygenated derivatives (xanthophylls e.g., lutein, α-cryptoxanthin, β-cryptoxanthin, and zeaxanthin) by all photosynthetic organisms including plants, algae, and cyanobacteria as well as by some non-photosynthetic bacteria and fungi (Armstrong 1994). Oxygen functions include hydroxylation at the 3- or 4- position (lutein and β-cryptoxanthin) and ketolation (canthaxanthin) as well as formation of the aldehydes, epoxy, carboxy, methoxy, and other oxygenated forms. Hydrocarbon carotenoids, carotenoid esters, and carotenoid fatty acid esters are frequently found in plant materials (Weeden and Moss 1995).The existence of uncommon or species-specific carotenoid has also been demonstrated. The most prominent examples are capsanthin and capsorubin, the predominant pigments of red pepper. Other classical examples of unique carotenoids are bixin, the major pigment of the food colorant annatto, and crocetin, the main coloring component of saffron (Rodriguez-Amaya 2001). In nature, carotenoids exist primarily in more stable all-*trans* isomeric form (Zechmeister 1962). The first two C_{40} carotenoids formed in the biosynthetic pathway have the 15-*cis* configuration in plants. The presence of small amounts of *cis* isomers of other carotenoids in natural sources has been increasingly reported (Rodriguez-Amaya 2001).

1.1. Plant carotenoids

Plant chloroplasts have a remarkably similar carotenoid composition with lutein (45% of the total), β-carotene (25–30%), violaxanthin (10–15%), and neoxanthin (10–15%)

as the most abundant carotenoids (Britton 1993). Most carotenoids are located, together with chlorophylls, in functional pigment-binding protein structures embedded in photosynthetic (thylakoid) membranes. While β-carotene is more abundant in the reaction centres of photosystems I and II, xanthophylls are preferentially distributed in the light-harvesting complexes that transfer excitation energy to the reaction centres (Demmig-Adams et al. 1996). Plant carotenoids are synthesized in chloroplasts. Biosynthesis of isoprenoid compounds and carotenoids are shown in Figures 1.1 and 1.2. Plants use both the methylerythritol phosphate (MEP) or 1-deoxy-d-xylulose-5-phopsphate (DOXP) pathway and the mevalonic acid (MVA) pathway for isoprenoid biosynthesis, although they are localized in different compartments. The MEP pathway synthesizes isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in plastids, whereas the MVA pathway produces cytosolic IPP. Mitochondrial isoprenoids are synthesized from MVA-derived IPP that is imported from the cytosol. Some exchange of IPP or a common downstream intermediate does also appear to take place between the plastids and the cytoplasm (Figure 1.1; Lichtenthaler et al. 1997; Lichtenthaler 1999; Rohmer 1999). Plant carotenoids have been classified as primary or secondary carotenoids. Primary carotenoids are the compounds required by plants in photosynthesis and function within the photosynthetic machinery (neoxanthin, violaxanthin, lutein, zeaxanthin and β-carotene). Secondary carotenoids are those carotenoids that are not exclusively required for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. They almost exclusively accumulate under stress conditions (e.g. nutrient starvation, salinity, high temperature etc.) and this process is species specific. The physiological function of secondary carotenoid remains to be clarified (Boussiba 2000). However, it is generally believed that they function as passive photoprotectants (i.e., as a filter) reducing the amount of light which can reach the light-harvesting pigment complex of PSII (Hagen et al. 1994).

Carotenoids serve at least two important functions in photosynthesis besides acting as membrane stabilizers. First, they participate in the light-harvesting process as accessory light-harvesting pigments that absorb light in the range of 450-470 nm and transfer the energy to chlorophyll. And second, they protect the photosynthetic apparatus from photo-oxidation by channeling excess energy away from chlorophyll (Bartley and Scolnik 1995). Carotenoids are essential for the efficient quenching of

chlorophyll triplets formed by an excess of light, preventing the generation of photo-oxidative species such as oxygen singlets that can react with lipids, proteins, and other macromolecules causing irreparable damage (Demmig-Adams et al. 1996). In addition, carotenoids dissipate excess light energy in a process known as thermal dissipation or non-photochemical quenching (NPQ) of chlorophyll fluorescence (Baroli and Niyogi 2000; Muller et al. 2001). Oxidative cleavage of carotenoids leads to the production of apocarotenoids, a structurally diverse class of compounds widely distributed in nature (Giuliano et al. 2003). Carotenoids have many industrial applications as natural food colorants (use of β-carotene in margarines), and used in cosmetic and pharmaceutical industries (Hirschberg 2001; Fraser and Bramley 2004).

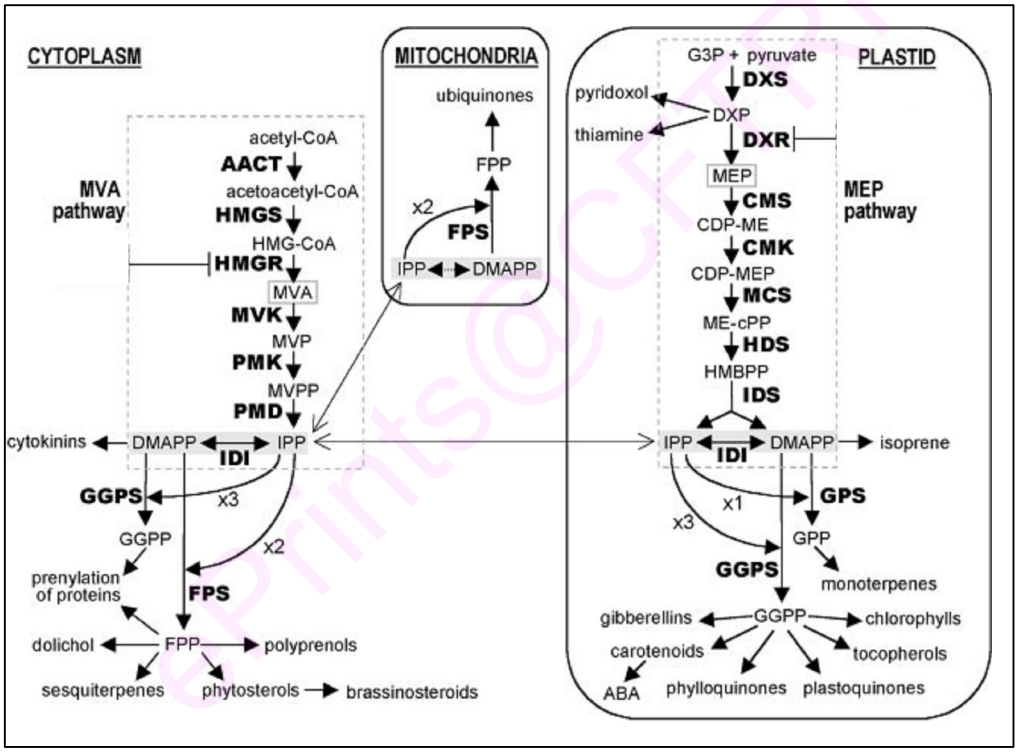


Figure 1.1. Isoprenoid biosynthesis pathways in the plant cell. HMG-CoA, Hydroxymethylglutaryl CoA; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; HBMPP,hydroxymethylbutenyl 4-diphosphate; FPP, farnesyl diphosphate; ABA, abscisic acid. The first intermediate specific to each pathway is boxed. Enzymes are indicated in bold: AACT, acetoacetyl CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MVK, MVA kinase; PMK, MVP kinase; PMD, MVPP decarboxylase; IDI, IPP isomerase; GPS, GPP synthase; FPS, FPP synthase; GGPS, GGPP synthase; DXS; DXR, DXP reductoisomerase; CMS; CMK; MCS; HDS; IDS, IPP/DMAPP synthase (Adopted from Rodríguez-Concepción and Boronat 2002).

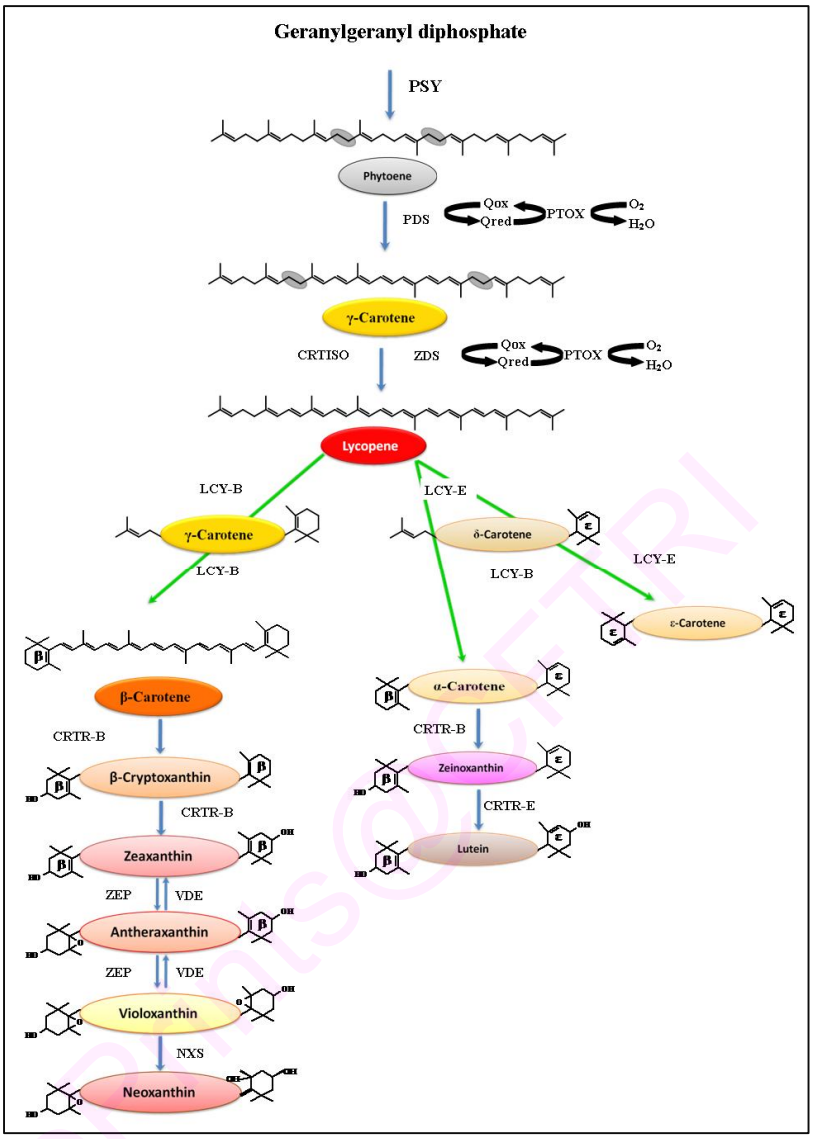


Figure 1.2. Overview of the biosynthesis of carotenoids in plastids. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; CRTR-B, β-carotene hydroxylase; CRTR-E, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, Violaxanthin de-epoxidase; NXS, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase (Adopted from Simkin et al. 2008).

1.2. Carotenoids in animals

Animals, including humans, do not synthesize carotenoids *de novo* and rely upon the diet. Dietary carotenoids give characteristic yellow to red colors to many birds, fish, and invertebrates and, more importantly they fulfill certain essential nutritional requirements of human beings and animals (Bollag 1996; Demmig-Adams and

Adams 2002; Fraser and Bramley 2004). Carotenoids act as precursors for the production of retinoids, and apocarotenoids synthesized in animal cells from carotenoids with β-ring end groups are taken from the diet (Bollag 1996). Retinoids such as retinol (vitamin A), retinal (the main visual pigment), and retinoic acid (which controls morphogenesis) play important functions as visual pigments and signaling molecules. The most potent dietary precursor of vitamin A is β-carotene. Its deficiency, reported as the most common dietary problem affecting children worldwide, leads to xerophthalmia, blindness, and premature death (Fraser and Bramley 2004). Although the major value of carotenoids in human nutrition is their role as provitamin A, recent studies support that their capacity of quenching singlet oxygen and acting as free radical scavengers and antioxidants *in vivo* can provide additional health benefits such as protection against macular degeneration, cardiovascular diseases and cancers (Handelman 2001; Demmig-Adams and Adams 2002; Johnson 2002; Fraser and Bramley 2004; Stahl and Sies 2005).

1.3. Carotenoids from microalgae

Microalgae produce wide spectrum of carotenoids. These carotenoids are associated with light capture in addition to chlorophyll. Carotenoids protect the microalgae against solar radiation and related effects. β-carotene from the alga *Dunaliella salina* is the first high value algal product commercialized which is now being produced in Australia, USA and Israel (Spolaore et al. 2006). Most of the chlorophycean members contains multitude of carotenoids- neoxanthin, violaxanthin, lutein, zeaxanthin, and antheraxanthin (Jin et al. 2003b). Due to its carotenoid rich nature, *Chlorella vulgaris* is being used as natural colour ingredient in animal feed (Gouveia et al. 1996). Few green algae like *Haematococcus, Chlorococcum, Neochloris* produce carotenoid astaxanthin along with other carotenoids. In Rhodophyta, the predominant carotenes are lutein, zeaxanthin, α and β-carotenes, while in Pheophyta, the main pigments are β-carotene, violaxanthin and fucoxanthin (Shahidi et al. 1998). Several carotenoids have been recognized as biotechnologically important (Table 1.1).

Table 1.1. Microalgal carotenoids of biotechnological importance

	Carotenoid Yield	References
Lutein		
Chlorella zofingiensis	21 µg ml^{-1}	Del Campo et al. (2004)
Chlorella protothecoides	225 µg ml^{-1}	Shi et al. (1999)
Muriellopsis sp.	35 µg ml^{-1}	Del Campo et al. (2001)
Zeaxanthin		
Dunaliella salina	6 mg g^{-1}	Jin et al. (2003a)
Microcystis aeruginosa	Not reported	Chen et al. (2005)
Nannochloropsis	Not reported	Lee et al. (2006)
Astaxanthin		
Haematococcus pluvialis	30 mg g^{-1}	Lorenz and Cysewski (2000)
Chlorella zofingiensis	<1 mg g^{-1}	Ip and Chen (2005)
β-**Carotene**		
Dunaliella salina	100 mg g^{-1}	Garcia-Gonzalez et al. (2005)
Dunaliella bardawil	>100 mg g^{-1}	Lers et al. (1990)
Canthaxanthin		
Chlorella emersonii	0.6 µg ml^{-1}	Arad et al. (1993)

Modified from Bhosale and Bernstein (2005)

1.4. Astaxanthin

Among the various commercially valuable compounds produced by microalgae, astaxanthin attracts a great commercial interest primarily due to its versatile applications and high price. Astaxanthin is a red pigment and oxygenated derivative of carotenoid (Figure 1.3), ketocarotenoid, employed as a pigmentation source in aquaculture (Lorenz and Cysewski 2000). It has also been shown to possess a higher antioxidation property than other carotenoids that may play an important role in cancer protection (Kobayashi et al. 1991). In the natural aquatic environment, astaxanthin is biosynthesized in the food chain within microalgae or phytoplankton at the primary production level. The microalgae are consumed by zooplankton, insects or crustaceans which accumulate astaxanthin and in turn, are ingested by salmonids. Astaxanthin cannot be synthesized by animals, hence must be acquired through diet. Since these organisms do not have access to natural sources of astaxanthin, the desirable reddish orange colour is imparted by feeding the astaxanthin rich diet. Adding to its pigmentation efficiency, astaxanthin has several essential biological

functions including protection against UV light effects, immune response and reproductive behaviour in the aquatic animals in which it is found (Lorenz and Cysewski 2000). This has led the researchers to explore the biological potentials of astaxanthin and prompted numerous research studies on its potential benefits to human beings and animals.

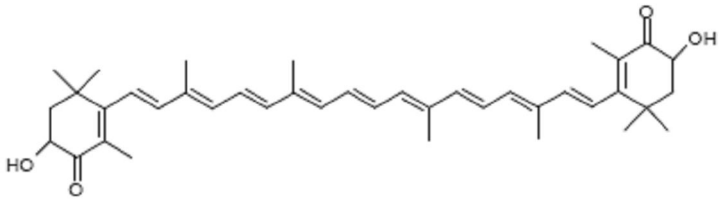


Figure 1.3. Structure of astaxanthin

1.5. The advantage of natural astaxanthin over synthetic astaxanthin

Astaxanthin is not only applied as feed additives for aquatic organisms but also as nutritional supplements for human beings. Natural astaxanthin is prevalently accepted by consumers for its food safety and health aspects (Krishna and Mohanty 1998; An et al. 1989; Lorenz and Cysewski 2000). Moreover, it should be mentioned that astaxanthin is a substance that contains a long hydrocarbon chain and if this is to be made artificially it involves many complicated chemical processes making it expensive with a market price of US$ 2500-3000 kg^{-1}. Therefore the search for less expensive natural sources of astaxanthin is necessary (Lorenz and Cysewski 2000; Dominguez-Bocanegra et al. 2004). Currently most of astaxanthin produced commercially is chemically synthesized. This synthetic pigment contains only 25% of the naturally occurring stereoisomer (3S, 3'S) although the biological significance of the chirality of astaxanthin is not known. In addition, chemically synthesized astaxanthin may be mixed with reaction intermediates (Morris et al. 2006).

1.6. Sources of astaxanthin

In nature, the main sources of astaxanthin are marine bacteria and microalgae. Fish and crustaceans, accumulate astaxanthin from their diet, resulting in characteristic colorations of flesh and/or carapace. Sources of astaxanthin are listed in Table 1.2. Production of astaxanthin from natural sources has been investigated and several

microorganisms are known to produce high levels of ketocarotenoids. Examples include the unicellular green alga *Haematococcus pluvialis*, an organism that can produce 4–5% ketocarotenoid on dry weight (Yuan and Chen 2000; Orosa et al. 2001). Another astaxanthin-producing microorganism is *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*), which can produce up to 0.5% dry weight astaxanthin, however this is in the 3R, 3'R form (Johnson and An 1991). Although uncommon, some plants do produce astaxanthin and other ketocarotenoids (Goodwin 1980; Czeczuga 1987). Most notably, the flowers of *Adonis aestivalis* accumulate ketocarotenoids at levels of up to 1% of dry weight (Renstrom et al. 1981). Recently, the enzymes involved in the conversion of β-carotene to astaxanthin have been characterized in *Adonis* (Cunningham and Gantt 2005).

Table 1.2. Natural sources of astaxanthin

Organism	Astaxanthin content (% w/w dry wt)	Reference
Green algae		
Haematococcus pluvialis	2.3-7.7	Kang et al. (2005)
Neochloris wimmeri	0.6	Orosa et al. (2000)
Chlorococcum	< 0.2	Zhang et al. (1997)
Nannochloropsis gaditana	<0.3	Lubian et al. (2000)
Scenedesmus vacuolatus	0.01	Orosa et al. (2000)
Chlorella zofingiensis	0.02-0.15	Ip and Chen (2005)
Chlamydomonas nivalis	0.04	Bidigare et al. (1993)
Monoraphidium sp. GK12	0.25	Fujii et al. (2008)
Scenedesmus komarekii	Not reported	Hanagata and Dubinsky (2002)
Fungi		
Xanthophyllomyces dendrorhous (Phaffia rhodozyma)	0.4	Jacobson et al. (2000)
Yeast-*Candida utilis*	0.04	Miura et al. (1998)
Bacteria		
Mycobacterium lacticola	0.003	Simpson et al. (1981)
Agrobacterium aurantiacum	0.01	Yokoyama et al. (1995)
Paracoccus carotinifaciens	Not reported	Tsubokura et al. (1999)
Brevibacterium sp	0.003	Neils and Leenheer (1991)
Animals		
Shrimp-*Pandalus clarkii*	0.015	Meyers and Bligh (1981)
Shrimp-*Pandalus borealis*	0.014	Shahidi and Synowiecki (1991)
Backs snow crab – *Chinoecetes opilio*	0.011	Shahidi and Synowiecki (1991)

Apart from these sources, some minor sources of astaxanthin include *Coalastrella striolata* (Abe et al. 2007) and *Chlamydomonas nivalis* where astaxanthin existed as glucoside esters (Řezanka et al. 2008).

1.7. Biotechnological significance of astaxanthin

1.7.1. Chemistry of astaxanthin

In nature, algae synthesize the carotenoid pigment astaxanthin which gets concentrated in the food chain through zooplankton and crustaceans, which are prey for salmon, trout and other aquatic animals (Steven 1948; Kitahara 1984; Foss et al. 1987). The astaxanthin molecule has two asymmetric carbons located at the 3 and 3' positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the way that the hydroxyl groups are attached to the carbon atoms at these centres of asymmetry. When the hydroxyl group is attached so that it projects above the plane of the molecule, it is said to be in the 'R configuration', and when the hydroxyl group is attached to project below the plane of the molecule, it is said to be in the 'S configuration'. Thus, the three possible enantiomers are designated: 3R,3R'; 3S,3S'; and 3R,3S' (meso) (Lorenz and Cysewski 2000).

Haematococcus primarily contains monoesters of astaxanthin linked to 16:0, 18:1 and 18:2 fatty acids. All of the free astaxanthin and its monoesters and diesters in *Haematococcus* have optically pure (3S, 3'S) chirality (Grung et al. 1992; Chien 1996). Fatty acids are esterified onto the 3' hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid, thereby increasing its solubility and stability in the cellular lipid environment. Studies now support a major role of astaxanthin in protecting *H. pluvialis* from oxidative stress (Kobayashi 2000; Li et al. 2008b). The composition of astaxanthin esters in *Haematococcus* is similar to that of crustaceans, the natural dietary source of salmonids (Storebakken et al. 1985).

1.7.2. Biological functions of astaxanthin

There is an increasing amount of evidence to suggest that astaxanthin surpasses the antioxidant benefits of β-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E. Studies have also shown that astaxanthin can protect skin from the damaging effects of ultraviolet radiation, ameliorate age-related macular

degeneration, protect against chemically induced cancers, increase high-density lipoproteins and enhance the immune system. Epidemiological studies have demonstrated a correlation between increased carotenoid intake and reduced incidence of coronary heart disease and certain cancers, macular degeneration and increased resistance to viral, bacterial, fungal and parasitic infections. Studies indicate that the mechanism for this protective attribute is partly owing to the direct enhancement of the immune response by carotenoids. Anti-carcinogenic effects of carotenoids are likely to be attributable to its antioxidant effect, in so much as oxygen radicals are related to the process of cancer initiation and propagation (see Lorenz and Cysewski 2000).

Numerous studies have demonstrated the potent radical scavenging and singlet oxygen quenching properties of astaxanthin. As a result of its particular molecular structure, astaxanthin has a potent neutralizing or 'quenching' effect against singlet oxygen, as well as a powerful scavenging ability for free radicals, and it serves as an extremely potent antioxidant against these reactive species. Within the cell, it can effectively scavenge lipid radicals and destroys peroxide chain reactions to protect fatty acids and sensitive membranes (Terao 1989; Kurashige et al. 1990; Miki 1991). It has been demonstrated that astaxanthin is significantly more effective than β-carotene in neutralizing free radicals and gives better protection against the peroxidation of unsaturated fatty acid methyl esters than canthaxanthin, β-carotene or zeaxanthin (Lee et al. 1986; Jorgensen and Skibsted 1993). The antioxidant activities of astaxanthin have been shown to be approximately ten times greater than other carotenoids, such as zeaxanthin, lutein, canthaxanthin and β-carotene, over 500 times greater than alpha-tocopherol, and astaxanthin has been proposed to be the 'super vitamin E' (Ranby and Rabek 1978; Miki 1991; Shimidzu et al. 1996).

Astaxanthin might also be useful in preventing age related macular degeneration (AMD), which causes irreversible blindness. When high-energy blue light waves interact with the retina, they can cause peroxide damage of the lipids through photo-oxidation, which in turn creates singlet oxygen and free radicals. Carotenoids within the macula absorb the high-energy blue light thereby quenching these damaging oxygen species. Clinical studies have indicated that light injury is a cause of AMD because of the cumulative insult leading to a gradual loss of photoreceptor cells. Unlike β -carotene, astaxanthin is able to readily cross the blood-retinal–brain barrier and can protect the retina against photo-oxidation and loss of

photoreceptor cells. Furthermore, astaxanthin has the ability to protect the neurons of the retina, as well as the central nervous system, especially the brain and spinal cord, from damage caused by free radicals.

It appears that astaxanthin can also decrease the oxidation of lipid carriers and thereby reduce the risk of atherosclerosis (Murillo 1992). In rat kidney fibroblasts, the addition of astaxanthin confers greater protection against ultraviolet (UV-A)-light-induced oxidative stress compared with lutein and β-carotene. When cell cultures were grown in carotenoid-supplemented media and exposed to UV-A light, β-carotene at a level of 1000 nM and lutein at 100 nM returned catalase activity to control levels, whereas it only required 5 nM of astaxanthin (O'Connor and O'Brien 1998).

1.7.3. Industrial applications of astaxanthin

Industrial applications of carotenoids include their use as colorants for human food, as feed additives to enhance the pigmentation of fish, eggs, cosmetics, and pharmaceutical products. The major market for astaxanthin is its use as a pigmentation source in aquaculture and poultry industries (Lorenz and Cysewski 2000). It specifically provides a characteristic pink color to salmonoids, trout and shrimp (Jin et al. 2006). Astaxanthin sells for around U.S. $2500/kg with an annual worldwide market estimated at over U.S. $200 million. The astaxanthin used by the fish farmers represents 10 to 20% of the feed cost. Although more than 95% of this market consumes synthetically derived astaxanthin, consumer demand for natural products makes the synthetic pigments less desirable and provides an opportunity for the production of natural astaxanthin by the alga *Haematococcus*. *Haematococcus* is a potential source producing 1.5 to 3.0% astaxanthin. It has already gained acceptance in aquaculture and other markets as a concentrated form of natural astaxanthin. The Canadian Food Inspection Agency and the U.S. FDA have approved the use of this alga as a color additive in salmonoid feeds. Astaxanthin is used in aquaculture as it has functions such as antioxidant, hormone precursor, immune enhancement, provitamin A, reproduction, growth, maturation, and photoprotection (Margalith 1999; Lorenz and Cysewski 2000). In general, astaxanthin is used as a nutraceutical ingredient. The algal meal has been approved as a natural red food color in Japan and some European countries as well as a dietary supplement ingredient in the United States (Lorenz and Cysewski 2000). Because of the ability of *H. pluvialis* to

accumulate higher amounts of astaxanthin and its multidimensional applications in various industries, it is recognized as a potential candidate for the production of astaxanthin.

1.8. Haematococcus pluvialis

1.8.1. Occurrence and distribution of *Haematococcus pluvialis*

The fresh-water unicellular green alga *Haematococcus pluvialis* Flotow, also referred to as *Haematococcus lacustris* or *Sphaerellla lacustris*, belongs to the Division Chlorophyta, Class Chlorophyceae, Order Volvocales, Family Haematococcaceae, Genus *Haematococcus* and Species *pluvialis* (Bold and Wynne 1985). *Haematococcus* is widely distributed and occurs primarily in ephemeral rain pools and birdbaths (Czygan 1970). It has been isolated across Europe, Africa and North America (Pringsheim 1966). Recent reports show its existence in small artificial pool in poland (Burchardt et al. 2006) and natural and man-made ponds in Himachal Pradesh, India (Suseela and Toppo 2006). The widespread occurrence of *Haematococcus* in temporary water bodies rather than permanent water bodies is in part due to the fact that such pools are usually free of other competing algae, and not to any inherent characteristic of the pools (Proctor 1957). *Haematococcus* is considerably well suited for survival under conditions of expeditious and extreme fluctuations in light, temperature and salt concentration than many other microalgae, due to its ability to encyst in a rapid manner (Proctor 1957).

1.8.2. Cellular morphology, ultrastructure, physiology and lifecycle of *Haematococcus pluvialis*

The life cycle of *H. pluvialis* consists of two phases – vegetative phase and astaxanthin accumulating phase (Kobayashi et al. 1991; Triki et al. 1997; Hagen et al. 2002; Sarada et al. 2002), and these phases can be divided into 5 stages namely, green vegetative flagellate cells, green resting vegetative cells, red flagellate cells, red cysts and germination of red cysts to vegetative cells (Figure 1.4). Motile vegetative flagellate cells along with resting vegetative cells are predominant in the culture under favorable conditions. The green flagellate cells are spherical to ellipsoid with a size between 8 to 20 µm and have two equal-length flagella emerging from anterior end. The cells contain a single cup-shaped chloroplast with several scattered pyrenoids.

The cells are characterized by a remarkable and distinct gelatinous extracellular matrix of variable thickness (Hagen et al. 2002). In its non-motile stage i.e., palmella stage, motile cells lose their flagella and become resting vegetative cells, accompanied by formation of an amorphous multilayered structure in the inner regions of the extracellular matrix or the primary wall (Hagen et al. 2002). Despite the loss of the flagella and the formation of the palmella membrane, the cellular structure remains the same as its motile form. When environmental conditions become adverse, i.e., nutrient deprivation, high light irradiance or high salinity, both motile and nonmotile vegetative cells transform into cysts or aplanospores (Santos and Mesquita 1984) and become resistant to prevailing extreme environmental conditions (Boussiba and Vonshak 1991). During the transformation, the algal cell quickly rounds up, looses its flagella, rendering the cyst cells immotile. The cell size increases up to 40µm in diameter and a trilaminar sheath and acetolysis resistant material-based secondary wall is formed and thickened. It also coincides with expansion of cell volume (Montsant et al. 2001) and massive accumulation of astaxanthin in lipid globules in the cytoplasm (Hagen et al. 2002). Under nutrient deficient and high light condition, the cells start accumulating astaxanthin without losing their flagella and not forming cysts. This cultivation scheme is having advantages, since fragile cell boundary of flagellates are more accessible to biochemical investigations including cell fractionation and electron microscopy as compared to cyst cells (Grünewald and Hagen 2000).

Vegetative phase cells possess chlorophyll a and b and primary carotenoids, β-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin as found in Chlorophyta and in the chloroplasts of higher plants (Harker et al. 1996). Under stress conditions, accumulation of astaxanthin is accompanied by a decrease in chlorophylls and primary carotenoids (Harker et al. 1996). Light and electron microscopic studies revealed that, in motile cells, astaxanthin first appears in small spherical inclusions (with no true limiting biomembrane) in the perinuclear cytoplasm, the pigment granules are not within any specific organelle or vesicle (Lang 1968; Santos and Mesquita 1984). In maturing cysts the pigment deposit increase in number and take on a variety of shapes. Coalescence of the globular granules result from increasing quantities of astaxanthin formed as the cell ages. In mature cysts the cytoplasm is almost uniformly red with reduced amounts of pigments in the chloroplast.

Astaxanthin disperses towards the periphery of *Haematococcus* cells under light induction and moves back towards the center after illumination is discontinued (Yong and Lee 1991).

Light microscopic studies of astaxanthin accumulation process in the flagellates of *H. pluvialis* cells revealed the occurrence of small orange red cytoplasmic vesicles in the perinuclear region (Grünewald and Hagen 2000). As the accumulation process progressed, the size and number of secondary carotenoid (SC) vesicles increased markedly and vesicles were found accumulated all over the cytoplasm. Changes in flagellates ultrastructure were visible. Besides reduction in number of thylakoids, a marked accumulation of starch in the chloroplast was notable two days after the onset of induction of astaxanthin biosynthesis. In following stages the amount of starch decreased quickly. Throughout the SC accumulation period the chloroplast envelope remained intact. Budding or release of vesicles from chloroplast envelope was not observed. However, numerous cases of budding and extrusion of large carotenoid containing lipid vesicles across the plasma membrane into the extracellular matrix with all intermediate stages were observed. Light microscopic observations also showed vesicles in and peripheral of the extracellular matrix of flagellates during massive accumulation of SC, about 4 days after the onset of carotenogenic conditions. Electron microscopic studies showed bulging of the plasmalemma and subsequent release of the large electron dense vesicles from the cytoplasm into the extracellular matrix, and extrusion of vesicles out of the extracellular matrix was never observed (Grünewald and Hagen 2000).

However, the process of cyst formation is reversible, i.e., when mature cysts are transferred to fresh medium and exposed to a low light, intracellular daughter cells are released from the mature cyst cells into the medium, and vegetative cells regenerated from daughter cells grow mixotrophically. Germination of cysts coincides with chlorophyll and protein synthesis, and carotenoid degradation (Fabregas et al. 2003).

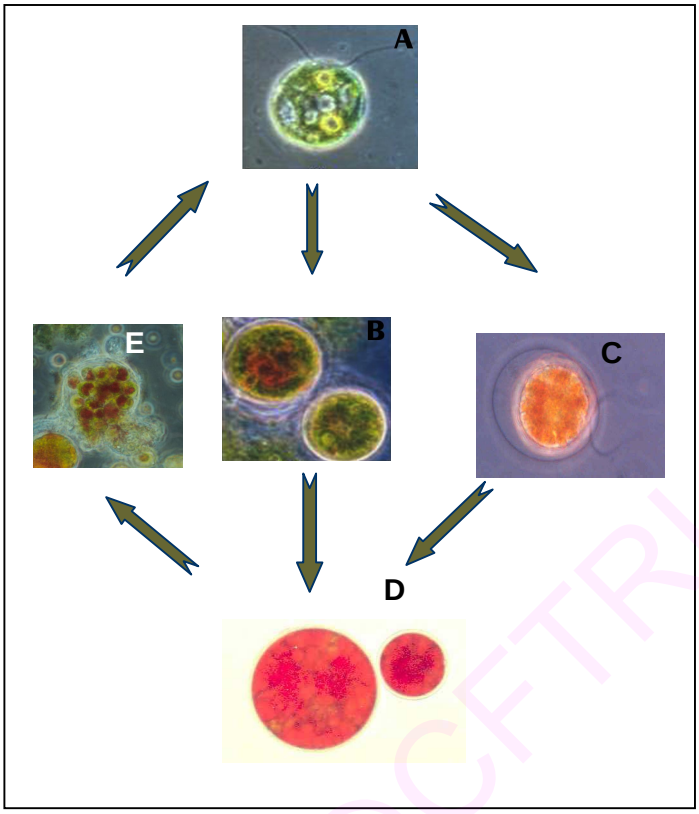


Figure 1.4. Life cycle of *H. pluvialis* showing green vegetative motile cells (A), vegetative palmella cells (B), astaxanthin accumulating flagellated cells (C), astaxanthin accumulating cyst cells (D) and germinating cells (E).

1.8.3. Stress conditions and accumulation of astaxanthin

Over the years studies were performed to understand the transformation of flagellates into red cyst cells, particularly the effect of light on astaxanthin accumulation. Goodwin and Jamikorn (1954) showed that light is necessary for astaxanthin synthesis, where as Droop (1955) has a opposing result showing that *H. pluvialis* can synthesize astaxanthin in dark in the presence of sodium acetate. Though the accumulation occurred slowly, the results were confirmed by adding various chlorides to heterotrophically grown cells in dark (Kobayashi et al. 1997b).

From earlier works it may be assumed that transformation of flagellates into red cyst cells is caused as a result of slowing down the cell division rate and /or the depletion of nutrients (Czygan 1970; Borowitzka et al. 1991; Zlotnik et al. 1993). Transfer of logarithmic phase cells to nitrogen deficient medium decreased cell division along with induction of astaxanthin synthesis and cyst cell formation. This assumption is in line with the previous experiment in which the cell division inhibitor vinblastin was able to induce astaxanthin formation in green vegetative cultures

(Boussiba and Vonshak 1991). The induction of astaxanthin accumulation is not necessarily a process associated with the cyst cell formation (Lee and Ding 1994; Hagen et al. 2000). The role of several environmental stress conditions on the induction and accumulation of astaxanthin has also been demonstrated by a number of researchers. Formation of reddish palmella cells and cysts can be induced in autotrophic growth condition under low nitrate or high phosphate concentrations (Borowitzka et al. 1991). Mixotrophic growth with acetate improves growth rate and final cell yield, and also stimulates the formation of the astaxanthin containing palmella cells and cysts (Borowitzka et al. 1991; Kobayashi et al. 1992). Formation of palmella cells and aplanospores can also be stimulated by increasing the temperature or by the addition of NaCl (Borowitzka et al. 1991). Boussiba et al. (1992) summarized the effect of higher light intensities, nitrogen limitation, phosphate or sulfate starvation or salt stress (0.8% NaCl) on the accumulation and cyst cell formation. The inhibition of the glutamine synthase by the herbicide BASTA (sodium glufosinate) also led to the accumulation of astaxanthin similar to the effect of nitrogen starvation (Aflalo et al. 1999).

1.8.4. Physiological and biochemical changes during astaxanthin accumulation

Research on the physiological changes occurring during astaxanthin accumulation in *H. pluvialis* is less. One of the important aspects studied was changes in lipid content. It is observed that increase in lipid content (Boussiba and Vonshak 1991) and oleic acid rich triacylglycerol (Zhekisheva et al. 2002) has a direct association with astaxanthin accumulation. In support of this, a steep increase in the content of all the three lipid classes viz., glycolipids, phospholipids and neutral lipids was reported in the cultures which accumulate astaxanthin after exposure to high light intensities. Especially the neutral lipids triacylglycerols were accumulated in high amounts, with oleic acid predominating. It is also observed that Inhibition of fatty acid synthesis has significant effect on astaxanthin biosynthesis, whereas the inhibition of carotenoid biosynthesis by norflurazon or diphenylamine (DPA) has only little effect on lipid synthesis (Zhekisheva et al. 2005). This suggests that the accumulation of astaxanthin is closely related to the synthesis of fatty acids. It is possible that the accumulated fatty acids serve as a matrix for solubilizing astaxanthin esters. The free non-esterified form of astaxanthin is found only in small amounts of 1% (Grung et al. 1992).

Vegetative green cells are dominated by chlorophyll a and b followed by the carotenoids lutein (59% of total carotenoids) and β-carotene (13.5 % of total carotenoids) and the xanthophyll violaxanthin (14.5% of total carotenoids) (Orset et al. 1995). Astaxanthin increases from few picogram per cell to a few hundreds when encystment is induced, at this stage astaxanthin esters constitute up to 98% of the total carotenoids and reach up to 4% of total cellular dry weight (Boussiba et al. 1999). The astaxanthin pool consist approximately 70% monoesters, 25% diesters and 5% free form (Lorenz and Cysewski 2000).

Contrary results were reported about the photosynthetic activity measured in terms of oxygen evolution rates during astaxanthin accumulation and encystment of *H. pluvialis* induced by nutrient deprivation under high light intensities. When vegetative cells were grown in nitrogen deficient medium and high light, the cyst cell formation was accompanied by massive accumulation of astaxanthin and a slight decrease in chlorophyll content from 16 to 14.8 pg $cell^{-1}$ (Zlotnik et al. 1993). The cyst cell formation was characterized by a gradual reduction in the maximal photosynthetic rate and increase in the photosynthetic quantum requirement and minimal turnover time for photosynthetic O_2 evolution. Respiration rate increased 4 times during aplanospore formation. Measurements of the cellular content of photosystem II (PSII) reaction centers suggest that the photosynthetic complex remains relatively stable during the formation process and in the mature aplanospore.

High light coupled with phosphate deficiency induced aplanospore formation showed significantly decreased level of cytochrome *f*, which impairs the linear electron flow from photosystem II to photosystem I. The respiration rate of red cells was higher than that of green cells, but the level of apoproteins, CPI, D2, CP47, LHCI were decreased in the red cells. Conversely the O_2 evolution was positive in green cells whereas it was negative in aplanospores. However, the activity of the photosynthetic electron transport was functional in red aplanospores (Tan et al. 1995).

In earlier studies, astaxanthin accumulation in *Haematococcus* cells were induced either by nutrient deficiency or by the use of aged cultures, in which the cell division slowed down or stopped with the reduction in photosynthetic activity and decomposition of photosynthetic apparatus. So the damage to the photosynthetic apparatus may be a side effect of the conditions used for the induction of astaxanthin accumulation but not directly related to this process (Wang et al. 2003b). To avoid the side effects by other stress conditions, only high light was applied for astaxanthin

induction and the induction process was accompanied by significant reduction in D1 protein (Wang et al. 2003b). However the decline in D1 content stopped after one day and recovered to normal after 5 days. The photosynthetic rates remained high and hence formation of astaxanthin seems to prevent further photo-oxidative damage. This in turn helps to maintain the PSII function and the structural integrity of photosystems and enables the cell to cope with high light condition.

Accumulation of a number of protease-resistant, heat-stable proteins with apparent molecular masses of 38 kDa, 50 kDa, 62 kDa and 63 kDa was reported in astaxanthin accumulating red cyst cells (Pelah et al. 2004). This protein fraction was effective in the protection of horseradish peroxidase from inactivation, suggesting a role for these proteins in *H. pluvialis* subjected to a stress event (Pelah et al. 2004).

1.8.5. Biosynthesis of astaxanthin in *H. pluvialis*

Structurally, carotenoids are tetraterpenes, derived from the 5-carbon units IPP and its isomer DMAPP (Cunningham and Gantt 1998). Plants synthesize IPP and DMAPP via two different pathways: the cytosolic MVA pathway and the MEP pathway that is localized within the chloroplast (Lichtenthaler 1999; Eisenreich et al. 2001). The inhibition by fosmidomycin of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), an enzyme of the plastidial MEP pathway, suppressed the synthesis of astaxanthin in *H.pluvialis* flagellates (Hagen and Grünewald 2000). This finding indicates that the MEP pathway provides IPP precursors for carotenoid biosynthesis in *H. pluvialis.*

Figure 1.5 schematically depicts the key steps in carotenoid biosynthesis pathway in *H. pluvialis.* The reversible isomerization reaction between IPP, a key building block of carotenoids, and its allylic isomer DMAPP is carried out by the enzyme isopentenyl pyrophosphate isomerase (IPI) (Figure 1.1). Two cDNAs for *IPI* gene have been identified in *H. pluvialis* (Sun et al. 1998). The first committed step in the biosynthesis of carotenoids, the head-to-head condensation of two 20-carbon geranylgeranyl diphosphate (GGPP) to 40-carbon molecule phytoene, is mediated by the soluble enzyme phytoene synthase (PSY) The subsequent steps of the pathway leading to the synthesis of colored carotenoids are carried out by desaturation and β-cyclization reactions catalyzed by membrane-localized enzymes such as phytoene desaturase (PDS) and lycopene β-cyclase (LCY) respectively (Figure 1.5;

Cunningham and Gantt 1998). The genes for ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) have not yet been reported in *Haematococcus*.

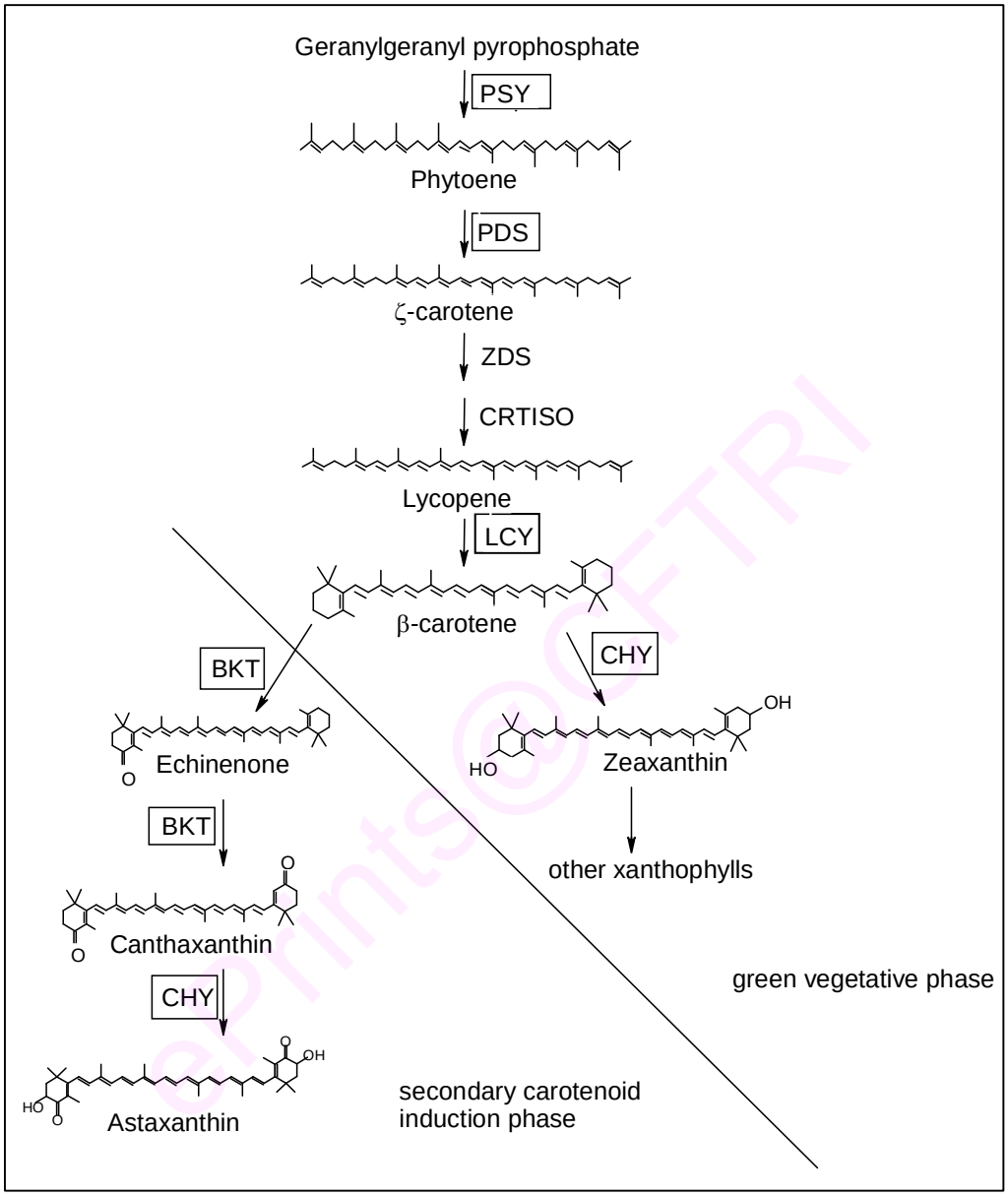


Figure 1.5. Biosynthetic pathway of astaxanthin in *H. pluvialis*

Although the specific steps of astaxanthin biosynthesis are carried out in the cytoplasm, the enzymes of the general carotenoid pathway appears to be localized in the chloroplast (Grunewald et al. 2000; Jin et al. 2006). The biosynthesis of astaxanthin in *H. pluvialis* follows the general carotenoid pathway up to β-carotene formation. From β-carotene, astaxanthin is formed by the action of two enzymes β-carotene ketolase (BKT) (synonym: β-carotene oxygenase, CRTO) and β-carotene hydroxylase (CHY or CRTR-B or CRTB). Two possible routes of astaxanthin

formation have been postulated: the first one follows formation of echinenone, canthaxanthin and adonirubin, whereas the second follows formation of β-cryptoxanthin, zeaxanthin and adonixanthin (Fraser et al. 1997; Grünewald et al. 2001). Studies using carotenogenic inhibitors and *in vitro* and *in vivo* analyses of astaxanthin synthesis in *Haematococcus* confirmed that astaxanthin is produced via formation of canthaxanthin. BKT converts β-carotene to canthaxanthin via echinenone which is further acted upon by CHY resulting in the formation of astaxanthin (Fan et al. 1995; Fraser et al. 1998; Schoefs et al. 2001).

1.8.6. Regulation of carotenogenesis in *H. pluvialis*

Regulation of carotenogenesis *in vivo* is not studied in detail, though the nucleus encoded genes for enzymes of the carotenoid biosynthetic pathway have been identified in several organisms including algae (Cunningham and Gantt 2000; Steinbrenner and Linden 2003; Yan et al. 2005; Zhu et al. 2005). However, studies on expression of several genes involved in carotenoid biosynthesis indicate that *Haematococcus* carotenoid biosynthesis genes are up-regulated at either mRNA and protein level, or both (Sun et al. 1998; Grünewald et al. 2000; Steinbrenner and Linden 2001). In general, all *Haematococcus* genes studied coding for enzymes involved in carotenoid biosynthesis were shown to be up-regulated at the mRNA level in response to environmental stress conditions. Regarding genes coding for enzymes involved in biosynthesis of carotenoid precursors, Sun et al. (1998) showed that both isopentenyl pyrophosphate isomerase (*IPI*) genes – *IPI1* and *IPI2* are up-regulated at mRNA level, and *IPI2* was found only in the cytosol which is enhanced at protein level also, suggesting that biosynthesis of carotenoid precursors are regulated at different levels – transcriptional, translational and post-translational.

PSY catalyzes the initial step in the carotenoid biosynthesis pathway, hence makes it a target for possible regulation of metabolic flux into the pathway. The mRNA of the *PSY* were up-regulated in response to various stress conditions (Steinbrenner and Linden 2001; Pulz and Gross 2004) and sodium acetate, Fe^{2+} and high light stress (Steinbrenner and Linden 2001), though the regulation of PSY at the protein level is unknown. Under SC inducing conditions, the enzyme PDS was induced at mRNA and protein level and PDS was found exclusively in the chloroplast (Grunewald et al. 2000), suggesting that the carotenoid precursors of astaxanthin are made in the chloroplast. Since astaxanthin accumulation occurs in lipid vesicles of the

cytoplasm, the transfer of carotenoids from chloroplast to cytoplasm must occur. The exposure of *Haematococcus* cells to stress conditions caused increase in mRNA levels of lycopene β-cyclase (LYCB) but the protein level of LYCB remained constant (Sun et al. 1996; Steinbrenner and Linden 2003). This has lead to the speculation that the LYCB enzyme present in non-stressed cells becomes more active when cells were exposed to stress conditions.

Astaxanthin was postulated to be biosynthesized by two slightly different pathways with both pathways involving the enzymes BKT and CHY. BKT was up-regulated both at mRNA level and protein level (Sun et al. 1996; Grünewald et al. 2000; Grünewald et al. 2001), but for CHY, up-regulation was demonstrated only at mRNA level (Linden 1999; Steinbrenner and Linden 2001; Steinbrenner and Linden 2003). Grunewald et al. (2001) showed that BKT is located both in chloroplast and cytosolic lipid vesicles. Moreover, BKT activity is demonstrated *in vitro* in the lipid vesicles, indicating that BKT acts not only in the chloroplast, but also in the cytosol. Multiple forms of BKT have been recently identified and found to be differentially expressed in response to stress conditions (Huang et al. 2006a).

Recent report showed that the regulation of gene expression under stress condition is linked to the redox state of the plastoquinone pool (Steinbrenner and Linden 2003). Blocking the photosynthetic electron transport chain at the level of plastoquinone reduction will either completely prevent or diminish the light stress induced up-regulation of carotenoid genes expression. The hypothesis that the stress response in *H. pluvialis* may be mediated by ROS is based on the report which showed that ROS generating compounds such as Fe^{2+}, methyl viologen and methylene blue resulted in increased astaxanthin accumulation (Kobayashi et al. 1993; Fan et al. 1998; Boussiba 2000). But Steinbrenner and Linden (2001) suggested that ROS generators are not involved in the transcriptional regulation of PSY and carotenoid hydroxylase. In corroboration with this finding, previous reports showed that the effect of Fe^{2+} on astaxanthin accumulation is independent of *de novo* protein biosynthesis and it is suggested that there is a function of ROS at the post-translational level (Kobayashi et al. 1993).

Some reports on kinetic analysis of enzymes involved in *H. pluvialis* astaxanthin biosynthesis suggested the requirement of oxygen, NADPH and Fe^{2+} for astaxanthin synthesis (Fraser et al. 1997; Cunningham and Gantt 1998; Fraser et al. 1998). It was proposed that cytochrome P450-dependent enzyme is involved in the

transformation of β-carotene to astaxanthin. The hydroxylase activity appears to be cytochrome P450 dependent, as evidenced by the accumulation of canthaxanthin when cytochrome P450 enzyme activity was inhibited by a specific inhibitor ellipticin (Schoefs et al. 2001). The BKT enzymes are not so defined in their substrate preference. However, the enzymes are strictly oxygen-requiring; and a cofactor mixture of 2-oxoglutarate, ascorbic acid, and Fe^{2+} is beneficial to activity.

The gene β-carotene oxygenase (CRTO) of *Chlorella zofingiensis* has been cloned and characterized and found to be transiently up-regulated upon glucose treatment (Huang et al. 2006b). In dark-grown *C. zofingiensis*, the transcription of *BKT* and *CHY* genes are differently regulated by the metabolism of glucose, through which the biosynthesis of astaxanthin is regulated. Phosphorylation of glucose (glucose sensing) was essential to the increased transcription of *BKT* and *CHY* genes and the accumulation of astaxanthin in the dark grown cells. However, phosphorylation of glucose *per se* only up-regulated the transcription of *CHY* and stimulated the synthesis of zeaxanthin. Blockage of the mitochondrial alternative pathway eliminated the glucose effects on the increased transcription of *BKT* and astaxanthin accumulation, suggesting that signals from alternative pathway was involved in the up-regulation of *BKT* transcription. In addition, citrate was shown to up-regulate the transcription of *BKT* independent of reactive oxygen species formation (Li et al. 2008a). Since glucose induced the expression of the *BKT*, effect of different sugars in regulation of carotenogenic genes expression was studied. The various sugars differentially regulated the transcription of the genes. Glucose and mannose, which supported the best growth and astaxanthin production of the algal cells, also resulted in the highest transcript levels of all the genes. Fructose, galactose, and sucrose only moderately enhanced the transcription of the genes; while lactose which was poorly metabolized by the cells did not up-regulate the transcription of the carotenogenic genes (Sun et al. 2008).

1.8.7. Differential expression of genes and proteins under conditions inducing secondary carotenoid production

Recent genomic and proteomic studies in *H. pluvialis* to investigate global gene and protein expression revealed that many genes and proteins related to growth, photosynthesis, and respiration are differentially expressed in addition to up-regulation of carotenoid genes and enzymes, during secondary carotenogenesis (Hu et

al. 2003; Wang et al. 2004a; 2004b; Eom et al. 2005). Such global analysis of carotenogenesis process will be helpful for identification of differentially expressed novel genes and gene products since it is possible that some of the gene products may be involved in regulation of the stress response mechanisms in *H. pluvialis*.

The protocol optimized by Wang et al. (2003a) for isolation of soluble proteins from microalgae using *H. pluvialis* as a model system was successfully applied to profiling protein expression and cell wall proteins under oxidative stress condition using two-dimensional gel electrophoresis. Seventy proteins with altered expression pattern were identified following stress induction to green flagellated cells by addition of acetate, Fe^{2+} and high light intensity exposure. Among these differentially expressed proteins, some key proteins involved in photosynthesis and nitrogen assimilation were down-regulated, whereas some mitochondrial respiratory proteins were transiently up-regulated after the onset of stress. Most of the identified proteins, particularly those from the families of superoxide dismutase, catalase, and peroxidase, were transiently up-regulated, but reverted to down-regulation during the 6 days of stress (Wang et al. 2004a). Kim et al. (2006) found up-regulation of 22 proteins over two-fold in red cyst cells when compared with the green vegetative cells. Further analysis of these proteins showed several key enzymes specific to the carotenoid pathway, including isopentenyl pyrophosphate isomerase and lycopene-β-cyclase appeared after exposure to high light intensity, which suggested involvement of these enzymes with carotenoid accumulation in the cytoplasm.

Analysis of cell wall proteins revealed that majority of identified cell wall proteins are differentially expressed at specific stages of the cell cycle along with a number of common wall-associated housekeeping proteins. Accumulation of cellulose synthase orthologue in the vegetative cells and putative cytokinin oxidase at the early stage of encystment were reported (Wang et al. 2004b). Proteomic analysis of cell wall-deficient mutants which were developed by chemical mutagenesis revealed that a majority of the cell wall proteins were present in the wild type and mutant cell walls throughout the cell cycle. Peptide mass fingerprinting (PMF) identified 55 wall protein orthologs from these mutants, including a subset of induced proteins known to be involved in wall construction, remodeling, and defense. Down-regulation of certain wall proteins in the two mutants was associated with the wall defects, whereas over expression of other proteins may have compensated for the defective walls in the two mutants (Wang et al. 2005).

Expressed sequence tags (ESTs) generated from a library constructed from astaxanthin-induced *H. pluvialis* revealed 63% of the EST sequences showed similarity to previously described sequences in public databases (Eom et al. 2005). *H. pluvialis* was found to consist of relatively high percentage of genes involved in genetic information processing (15%) and metabolism (11%), and low percentage of sequences were involved in the signal transduction (3%), structure (2%), and environmental information process (3%). In addition, a relatively large fraction of *H. pluvialis* sequences was classified as genes involved in photosynthesis (9%) and cellular process (9%). Based on this EST analysis, the full length cDNA sequence for superoxide dismutase was cloned, and the expression of this gene was changed substantially in response to different culture conditions, indicating the possible regulation of this gene in *H. pluvialis* (Eom et al. 2005). Analysis of cDNA microarray revealed differential expression of over 144 genes with a minimum of two-fold changes in red cyst cells produced by exposure of green vegetative cells to high irradiance and nutrient deprivation. There is a significant decrease in the expression of photosynthesis-related genes in cyst cells while the expression of defense or stress related genes and signal transduction genes were induced (Eom et al. 2006).

Differential display technique of Liang and Pardee (1992) was applied to identify genes that are specifically expressed under stress in *Haematococcus*. Isolation and characterization of one such RNA sequence was coding for *Haematococcus htrA*, a member of a heat shock serine protease family previously described only in prokaryotes. In *H. pluvialis*, transcripts of *htrA* were found to be differentially spliced, and the different splice products were differentially expressed during the developmental process. These transcripts were not detectable in vegetative cells, but were found at higher levels in developing aplanospores (Hershkovits et al. 1997).

1.9. Metabolic engineering of astaxanthin biosynthesis

Cloning of carotenoid biosynthetic genes is proved to be an essential tool for genetic manipulation of the pathway in plants to achieve higher carotenoid content or better carotenoid composition. The prospects of accomplishing these goals through gene transfer techniques seem to be high in view of the fact that transcriptional regulation of the biosynthetic genes is the major mechanism that regulates the pathway. A

significant accomplishment in the metabolic engineering of carotenoids towards improving the nutritional value of a major crop has been achieved in rice (*Oryza sativa*) and canola (*Brassica napus*) (Shewmaker et al. 1999; Ye et al. 2000; Sandmann 2001). *In vivo* production of astaxanthin and other ketocarotenoids by organisms that do not synthesize it in a natural way has been achieved by metabolic engineering in *Escherichia coli* (Lotan and Hirschberg 1995; Breitenbach et al. 1996), cyanobacteria (Harker and Hirschberg 1997) and several higher plants (Mann et al. 2000; Stalberg et al. 2003; Ralley and Fraser 2004; Gerjets and Sandmann 2006; Morris et al. 2006). All these transgenic hosts expressed *bkt* or *BKT* genes from *H. pluvialis*, *crtW* genes from several strains of bacteria or *CrtO* gene from cyanobacteria.

The gene *crtO*, which encodes β-C-4-oxygenase, a key enzyme in keto-carotenoid synthesis, was cloned from the alga *H. pluvialis*. Expression of the algal *CrtO* gene in the *Synechococcus* PCC7942, which normally synthesizes β-carotene and zeaxanthin, enabled the cyanobacterial cells to produce astaxanthin along with other keto-carotenoids (Harker and Hirschberg 1997). This result confirmed that *crtO* can function in conjunction with the intrinsic β-carotene hydroxylase that exists in all plants and algae to produce novel carotenoid species. Transformed tobacco plants that expressed *CrtO* in a regulated manner accumulated a high concentration of ketocarotenoids, including astaxanthin, in the chromoplasts of the nectary tissue in the flowers, changing their colour from yellow to red (Mann et al. 2000). As expected, the astaxanthin molecule that is synthesized artificially in the nectary occurs in the "natural" molecular configuration of the stereoisomer 3S,3'S. This feature distinguishes the astaxanthin produced in plants from the synthetic pigment, which is obtained as a mixture of molecules where 75% have different chiralities than the natural (3S,3'S) astaxanthin (Mann et al. 2000). The biological significance of the chirality of astaxanthin is not known. In the nectarines of transgenic plants, canthaxanthin and adonirubin are more abundant than zeaxanthin and adonixanthin, suggesting that the β-carotene ketolase (CRTO) preferentially uses β-carotene as a substrate and is more active than the hydroxylase. Trace amounts of ketocarotenoids found in the leaves of transgenic plants can be explained by the relatively low expression of *CRTO*, driven by the tomato *PDS* promoter, as indicated from the GUS assay. Unavailability of proper substrates for the β-carotene ketolase in chloroplasts

and lack of the ketocarotenoid-accumulating mechanism, i.e. binding proteins and lipid globules, could also affect astaxanthin formation in plant cells.

Transformation of *Arabidopsis* with an algal β-carotene oxygenase also leads to the accumulation of ketocarotenoids (Stalberg et al. 2003). In this case the major ketocarotenoids were 4-keto-lutein, adonirubin and canthaxanthin. In order to enhance the levels of ketocarotenoids, crosses were made with transgenic *Arabidopsis* over-expressing an endogenous phytoene synthase gene (Lindgren et al. 2003), resulting in an increase in ketocarotenoid content of up to 13-fold over that measured in the single β-carotene oxygenase transgenics. Recent studies demonstrated the accumulation of astaxanthin in plant storage organs. *H. pluvialis bkt1* encoding β-carotene ketolase was expressed in potato tubers (both *Solanum tuberosum* and *S. phureja*). In the transgenic tubers, two major ketocarotenoids, ketolutein and astaxanthin were detected and the level of unesterified astaxanthin reached ca. 14 μg g^{-1} DW in some *bkt1* expressing lines of *S. phureja* but was much lower in the less β-carotene producing *S. tuberosum* background (Morris et al. 2006). Transgenic carrot plants which are engineered for ketocarotenoid biosynthetic pathway are produced by introducing *H. pluvialis* β-carotene ketolase gene. Transgenic lines showed up-regulation of endogenous β-carotene hydroxylase expression in transgenic leaves and roots, and up to 70% of total carotenoids was converted to novel ketocarotenoids, with accumulation up to 2,400 μg g^{-1} root DW. Astaxanthin, adonirubin, and canthaxanthin were most prevalent, followed by echinenone, adonixanthin and β-cryptoxanthin (Jayaraj et al. 2008). The unicellular chlorophyte *Chlamydomonas reinhardtii* has been engineered with *bkt1* of *H. pluvialis*. A typical ketocarotenoid, 4-ketolutein was observed in all transformants, and no astaxanthin or canthaxanthin was observed (Leon et al. 2007). The gene coding for phytoene desaturase of *H. pluvialis* was modified by site-directed mutagenesis, by changing the leucine codon at position 504 to an arginine codon. Modified *PDS* was integrated into nuclear genome of *H. pluvialis*. One of the transformants showed accelerated accumulation of astaxanthin compared to wild type upon stress induction (Steinbrenner and Sandmann 2006). These results demonstrate the prospect and potential of genetic engineering of ketocarotenoid biosynthesis for the production of commercially valuable compound, astaxanthin in plants.

Chapter II

Expression of Carotenoid Biosynthetic Genes and Carotenoid Accumulation

Summary

Haematococcus pluvialis, a green alga, accumulates carotenoids, predominantly astaxanthin, when exposed to stress conditions. In the present work, changes in the pigment profile and expression of carotenogenic genes under various nutrient stress conditions and their regulation were studied. Nutrient stress (modified BBM containing $1/10^{th}$ of N and P) and higher light intensity (60 µmol m^{-2} s^{-1}) in combination with NaCl/sodium acetate (SA) enhanced total carotenoid and total astaxanthin content to 32.0 and 24.5 mg g^{-1} of dry biomass, respectively. Expression of carotenogenic genes, phytoene synthase (*PSY*), phytoene desaturase (*PDS*), lycopene cyclase (*LCY*), β-carotene ketolase (*BKT*) and β-carotene hydroxylase (*CHY*) were up-regulated under all the stress conditions studied. However, the extent of expression of carotenogenic genes varied with stress conditions. Nutrient stress and high light intensity induced expression of *BKT* and *CHY*, astaxanthin biosynthetic genes transiently. Enhanced expression of these genes was observed with SA and NaCl/SA, while expression was delayed with NaCl. Maximum transcript levels of *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* were found to be 158–277, 5–9, 470–674, 28–40 and 451–673-fold higher, respectively, than green vegetative cells. The maximum content of astaxanthin recorded in cells grown in medium with SA and NaCl/SA correlated with the expression profile of the astaxanthin biosynthetic genes. Studies using various inhibitors indicated that general carotenogenesis and secondary carotenoid induction were regulated at both the transcriptional and the cytoplasmic translational levels. The induction of general carotenoid synthesis genes was independent of cytoplasmic protein synthesis while *BKT* gene expression was dependent on *de novo* protein synthesis. The present study also suggests a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes.

2.1. Introduction

Astaxanthin **(**3,3'-dihydroxy-β,β-carotene-4,4'-dione), is a commercially important, high value ketocarotenoid, used as a pigmentation source in the aquaculture and poultry industries. It has also found applications in the nutraceutical, pharmaceutical, and cosmetic industries (Guerin et al. 2003; Jin et al. 2006). *Haematococcus pluvialis* is a commercially promising source among the astaxanthin-producing organisms, owing to its ability to accumulate astaxanthin up to 4% (w/w) on a dry weight basis (Boussiba 2000). It accumulates astaxanthin in response to stress conditions such as high light intensity, salinity, acetate addition, nutrient depletion, influence of reactive oxygen species (ROS), and increased C/N ratio (Kobayashi et al. 1993; Sarada et al. 2002).

Carotenoids are ubiquitous and essential components of the photosynthetic tissues in plants, algae, and cyanobacteria wherein they participate in the light-harvesting process and protect the photosynthetic apparatus from photo-oxidative damage (Bartley and Scolnik 1995). In *Haematococcus*, astaxanthin accumulation occurs in extra-plastidic lipid globules as a secondary carotenoid (Grünewald et al. 2001). In the biosynthesis of carotenoids, the first committed step, the head-to-head condensation of geranylgeranyl diphosphate (GGPP) to phytoene, is mediated by the soluble enzyme phytoene synthase (PSY) (Figure 1.5). The subsequent steps of the pathway leading to the synthesis of colored carotenoids are carried out by membrane-localized enzymes such as phytoene desaturase (PDS) and lycopene β-cyclase (LCY) (Cunningham and Gantt 1998). The biosynthesis of astaxanthin in *Haematococcus* follows the general carotenoid pathway up to β-carotene formation. Studies using carotenogenic inhibitors and *in vitro* and *in vivo* analyses of astaxanthin synthesis in *Haematococcus* revealed the involvement of two enzymes β-carotene ketolase (BKT) (synonym: β-carotene oxygenase, CRTO) and β-carotene hydroxylase (CHY or CRTR-B) in the conversion of β-carotene to astaxanthin. BKT converts β-carotene to canthaxanthin via echinenone which is further acted upon by CHY resulting in the formation of astaxanthin (Fan et al. 1995; Lotan and Hirschberg 1995; Fraser et al. 1998). The genes for ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) have not been reported in *Haematococcus*. Although the specific steps of astaxanthin biosynthesis are carried out in the cytoplasm, the enzymes of the general

carotenoid pathway appear to be localized in the chloroplast (Grünewald et al. 2000; Jin et al. 2006).

Expression of carotenogenic genes has been studied in several plants including green algae, and transcription of carotenogenic genes were shown to be up-regulated by light (Bohne and Linden 2002; Simkin et al. 2003; Steinbrenner and Linden 2003; Romer and Fraser 2005) or a combination of light with N-deprivation (Grünewald et al. 2000). Although there are reports available on the influence of light on the expression of carotenogenic genes in *Haematococcus*, studies on the influences of other stresses on the expression of carotenogenic genes are limited (Grünewald et al. 2000; Huang et al. 2006a). The life cycle of *H. pluvialis* contains two distinct phases, namely a green motile vegetative phase and a non-motile astaxanthin-accumulating cyst phase (Sarada et al. 2002). This favours the use of *Haematococcus* as a model system to study the regulation of secondary carotenogenesis. Under nutrient-limiting conditions, the induction of astaxanthin accumulation occurs in flagellated cell (Brinda et al. 2004) which is more advantageous for biochemical analysis and the extraction of pigments as the cells are fragile. Understanding the molecular basis of stress-induced astaxanthin accumulation in *Haematococcus* will be useful for the optimization of astaxanthin production. Therefore, the present work focused on establishing a relationship between pigment profile and the corresponding expression profile of carotenogenic genes under the influence of nutrient depletion combined with high light intensity, and sodium acetate and NaCl. In order to understand the regulation of formation of general and secondary carotenoids, transcriptional and translational inhibitors were used.

2.2. Materials and Methods

2.2.1. Green alga *Haematococcus pluvialis*

The *Haematococcus pluvialis* (SAG-19a) culture was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institüt, Universität Göttingen, Göttingen, Germany.

2.2.2. Maintenance of stock culture

Stock cultures of *H. pluvialis* were maintained on both semisolid and liquid autotrophic Bold's basal medium (BBM) (Kanz and Bold 1969). The composition of the BBM is provided in Table 2.1.

Table 2.1. Composition of media for *H. pluvialis* growth

Constituents (g L^{-1})	BBM	Modified BBM	Nutrient limiting medium
$FeSO_4.7H_2O$	0.005	0.005	0.005
$CaCI_2.2H_2O$	0.024	0.024	0.024
$NaNO_3$	0.25	-	-
Ammonium carbonate	-	0.159	0.0159
$MgSO_4.7H_2O$	0.073	0.073	0.073
EDTA	0.045	0.045	0.045
K_2HPO_4	0.074	0.074	0.0074
KH_2PO_4	0.175	0.175	0.0175
Trace elements (Table 2.2)	1 ml	1 ml	1 ml

The ingredients were dissolved in distilled water and the pH was adjusted to 7.0 using 0.1N HCl and 0.1N NaOH. The above medium was solidified with agar (15 g L^{-1}) to prepare slants in test tubes. The liquid medium was distributed into 150 ml conical flasks, closed with cotton plugs and sterilized by autoclaving at 121°C for 20 min and allowed to cool at room temperature before inoculation. Inoculation was carried out under aseptic conditions in laminar air flow hood. The inoculated slant and liquid cultures were incubated in culture room under controlled temperature at 25±1°C and light intensity of 30 µmol m^{-2} s^{-1} under 16/8 h light/dark cycle. Light was provided by cool white fluorescent set of lamps (40W; Philips India Ltd, Kolkata,

India) and the light intensity was measured using lux meter (TES 1332, Taiwan). The liquid cultures were shaken manually once a day. The *H. pluvialis* slants and the liquid cultures were subcultured at every 4 week and 2 week intervals respectively.

Table 2.2. Composition of trace elements for *H. pluvialis* growth

Constituents	g 100ml^{-1}
H_3BO_3	0.3100
$MnSO_4.4H_2O$	0.2230
$ZnSO_4.7H_2O$	0.0287
$(NH_4)_6MoO_{24}.4H_2O$	0.0088
$(CoNO_3)_2.4H2O$	0.0146
$Na_2WO_4.2H_2O$	0.0033
KBr	0.0119
KI	0.0083
$Cd(NO_3)_2.4H_2O$	0.0154
$NiSO_4(NH_4)_2SO_4.6H_2O$	0.0198
$VoSO_4.2H_2O$	0.0020
$AlCl_3.6H_2O$	0.00237

2.2.3. Growth of *H. pluvialis*

The green motile vegetative cells were inoculated into liquid modified BBM medium (Table 2.1) and incubated in culture room under controlled temperature at 25±1°C and light intensity of 30 µmol m^{-2} s^{-1} under 16/8 h light/dark cycle for a period of 7 days. The liquid cultures were shaken manually once a day. The green motile cells thus produced were used for experiments.

2.2.4. Stress conditions and inhibitors

The cultures grown in modified BBM medium for 7 d were harvested by centrifugation at 3500 × *g* for 5 min and resuspended in fresh nutrient-limiting medium (Table 2.1) containing 1/10 of the nitrogen and phosphorus of the original medium. The initial cell concentration was adjusted to 15×10^4 cells ml^{-1}. The cultures were subjected to the following treatments (i) control (without NaCl and SA addition), (ii) NaCl 17.1 mM, (iii) sodium acetate 4.4 mM (SA), and (iv) NaCl 17.1 mM and SA 4.4 mM (NaCl/SA). The cultures were then incubated under a continuous light

intensity of 60 µmol m^{-2} s^{-1}. The transcriptional inhibitor actinomycin D (SRL, Mumbai, India), the cytoplasmic translational inhibitor, cycloheximide (Sigma-Aldrich, Bangalore, India), and the organellar translational inhibitor, chloramphenicol (HiMedia, Mumbai, India) were added at concentrations of 10 µg ml^{-1}, 300 ng ml^{-1}, and 50 µg ml^{-1}, respectively at the time of stress induction.

2.2.5. Growth measurement

2.2.5.1. Cell number

Algal cell number was determined by counting algal cells using Neubauer haemacytometer (Thoma neu, Germany) and expressed as number of cells ml^{-1}.

2.2.5.2. Dry weight

Known volume of culture was centrifuged at 3000 × *g* for 10 min and the algal biomass was washed with distilled water and dried in a hot air oven (Sanyo, Electrical Biomedical Co. Ltd., Japan) at 60°C till constant weight was obtained. Biomass weight was expressed as g L^{-1}.

2.2.6. Extraction and estimation of pigments

2.2.6.1. Pigment extraction

For pigment analysis, an aliquot of culture was harvested by centrifugation at 3000 × g for 10 min and freeze-dried. A known quantity of biomass was extracted with 90% acetone in a mortar and pestle using neutralized sand. The extraction was repeated till the pellet became colorless. The extracts were centrifuged at 8800 × g for 5 min and the supernatants were pooled. Aliquot of extract is flushed with N_2 gas and stored at -20°C preferably at -80°C for spectrophotometric analysis, and another aliquot of extract was evaporated to dryness using N_2 gas and stored at -20°C or -80°C for TLC and HPLC analysis. All operations were carried out under dim light

2.2.6.2. Spectrophotometric estimation of pigments

The acetone extracts absorbance was recorded at 470, 480, 645 and 661.5 nm using spectrophotometer (Shimadzu 160A). Chlorophyll and total carotenoid contents were calculated using following Lichtenthaler (1987) equations.

Chlorophyll a Chl a ($\mu g\ ml^{-1}$) = 11.24 $OD_{661.5}$ – 2.04 OD_{645}

Chlorophyll b Chl b ($\mu g\ ml^{-1}$) = 20.13 OD_{645} – 4.19 $OD_{661.5}$

Total Chlorophyll Chl a+b ($\mu g\ ml^{-1}$) = 7.05 $OD_{661.5}$ + 18.09 OD_{645}

Total carotenoid ($\mu g\ ml^{-1}$) = [1000 × OD_{470} - (1.9 × Chl a + 63.14 × Chl b)]/214

Pigment ($mg\ g^{-1}$) = (Pigment content ($\mu g\ ml^{-1}$) × volume of extract × dilution factor) / biomass taken for extraction (mg)

Astaxanthin content was determined at 480 nm using an absorption coefficient, A1% of 2500 by the method of Davies (1976).

Astaxanthin content (mg) = (OD_{480} × volume of extract × dilution factor × 10)/2500

2.2.6.3. Separation of carotenoids by thin-layer chromatography (TLC)

The *H. pluvialis* extracts were analysed using Silica Gel 60 F254 TLC plates (10 × 10 cm, Merck, New Delhi). *H. pluvialis* extract was spotted on TLC sheet and developed using solvent system acetone:hexane (3:7; Fiksdahl et al. 1978). The developed plates were allowed to dry at room temperature and carotenoids were identified by comparing with authentic β-carotene and astaxanthin standards (Sigma-Aldrich, St. Louis, MO, USA). Resolution front (Rf) value for individual bands and standards were calculated: Rf = distance traveled by analyte/distance traveled by solvent from origin.

2.2.6.4. High Performance Liquid Chromatograph (HPLC) of pigments

The *H. pluvialis* extracts were subjected to HPLC analysis in Shimadzu LC-10AT liquid chromatograph instrument using reverse phase C18 column (Supelco, 25 cm × 4.6 mm). The following solvents were used at a flow rate of 1.25 ml min^{-1}: (A) acetone and (B) 90 % methanol. The separation of carotenoids was achieved by a gradient between solvent A and B for 40 min as follows: B was run at 80 to 20% for 25 min, 20% for 10 min and 20 to 80% for 5 min. The separated carotenoids were identified by comparing retention times and spectra against known standards. Echinenone and astaxanthin esters (mono and di) were identified using a photodiode array detector (SPD-M10AVP, Shimadzu) by comparing their spectra and retention time with published data (Yuan and Chen 1998; Grünewald and Hagen 2001; Miao et al. 2006). The peaks were integrated by Class VP version 6.14 SP1 software (Shimadzu, Singapore) at 476 nm to quantify ketocarotenoids and 445nm to quantify

other carotenoids. The peaks were also integrated at 645nm to detect chlorophylls. Standard β-carotene, Lutein and astaxanthin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and canthaxanthin was obtained from ChromaDex, Inc. (SantaAna, CA, USA). Neoxanthin and Violaxanthin were gift from Dr. Akhihiko Nagao of Food Research Institute, Tsukuba, Japan.

2.2.7. RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

All glasswares and plasticwares used for RNA extraction and analysis were treated with 0.1% DEPC solution. Reagents used were either DEPC treated or filter sterilized. 1×10^8 cells were harvested from green motile cells and cells at different stages of stress induction, frozen under liquid nitrogen and subsequently powdered using a mortar and pestle. Then total RNA was extracted using RNAqueous® kit according to instruction manual (Ambion, Austin, TX). Possible contaminant genomic DNA in RNA extract was removed using TURBO DNA-free™ kit (Ambion, Austin, TX). The concentration of total RNA was determined spectrophotometrically at 260 nm. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide.

Table 2.3. Specific primers, annealing temperatures, and total numbers of amplification cycles used for RT-PCR

Primer	Primer sequence (5'–3')	Annealing temperature (°C)	Total number of amplification cycles	GenBank ID / reference	Amplicon size (bp)
PSY- forward	ATGTACCATCCCAAGGCAAG	60	30	AY835634	402
PSY- reverse	CTGGACCAGGCCTACGAC				
PDS- forward	TCCATGATCTTTGCCATGC	60	30	AY768691	462
PDS- reverse	CGGGAGTTGAACATGAGGTC				
LCY- forward	CTTCTTCTCCGCCTTCTTCA	60	30	AY182008	565
LCY- reverse	GCATCCTACCGCTCAAAGAA				
BKT- forward	CATCTCCTTGTACGCCTGGT	55	30	X86782	423
BKT- reverse	CAGTGCAGGTCGAAGTGGTA				
CHY- forward	CTACACCACAGCGGCAAGTA	55	30	AF162276	521
CHY- reverse	GCCTCACCTGATCCTACCAA				
Actin-forward	AGCGGGAGATAGTGCGGGACA	61	22	Huang et al. 2006a	200
Actin-reverse	ATGCCCACCGCCTCCATGC				

The gene specific primers for the genes *PSY, PDS, LCY, BKT* and *CHY* were designed using Primer3 software (Table 2.3) and synthesized (Sigma - Genosys, Bangalore, India), and for *Actin*, reported primers (Huang et al. 2006a) were used. First-strand cDNAs were synthesized from 1.5 µg of total RNA in 20 µl final volume, using M-MuLV reverse transcriptase and oligo-dT (18 mer) primer (Fermentas GmbH, Germany).

PCR amplifications were performed using PCR mixture (15 µl) which contained 1 µl of RT reaction product as template, 1× PCR buffer, 200 µM dNTPs (Fermentas GmbH, Germany), 1 unit (U) of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 or 22 cycles (1 min at 94°C; 1 min at 55 or 60°C; 1 min at 94°C) and final elongation (10 min at 72°C) using a thermal cycler (Eppendorf Thermal cycler, Germany). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%) and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GmbH, Germany). The band intensity of each gel was checked using the Herolab E.A.S.Y Win 32 software (Herolab GmbH Laborgerate, Germany). Each band was normalized against the intensity obtained with the same cDNA using the *Actin*-specific primers, whose expression was constant under all culture conditions (Eom et al. 2005; Huang et al. 2006a). For calculating the transcript abundance under stress conditions, the transcript levels of each gene in green motile cells were taken for comparison, while under inhibitor-added conditions, transcripts of the respective control (without inhibitors) were used for comparison.

2.2.8. Experimental design and data analysis

Each experiment was repeated three times with at least three replications. All the observations and calculations were made separately for each set of experiments and were expressed as mean±SD. The significance ($p \leq 0.05$) of the variables studied was assessed by one-way analysis of variance (ANOVA) using Microsoft Excel XP®. The mean separations were performed by Duncan's Multiple Range Test (DMRT) for segregating means where the level of significance was set at $p \leq 0.05$ (Harter 1960).

2.3. Results

2.3.1. *Haematococcus pluvialis* growth under nutrient stress and continuous high light

Exposure of *H. pluvialis* to stress conditions increased the number of cells produced. Cells exposed to nutrient stress and high light retained their flagella for longer periods whereas the addition of NaCl and SA induced early cyst formation. On the second day of stress induction, 18, 40, 82, and 89% of cells were transformed to cysts in control, NaCl-, SA-, and NaCl/SA-added cultures respectively. Nine days after stress induction, 42% of cells in control culture retained flagella whereas cells in NaCl-,SA-, and NaCl/SA-added cultures lost their flagella completely. As the stress advanced, all the treatments showed an increase in biomass yield and the SA- and NaCl/SA-added cultures produced significantly more biomass (Figure 2.1).

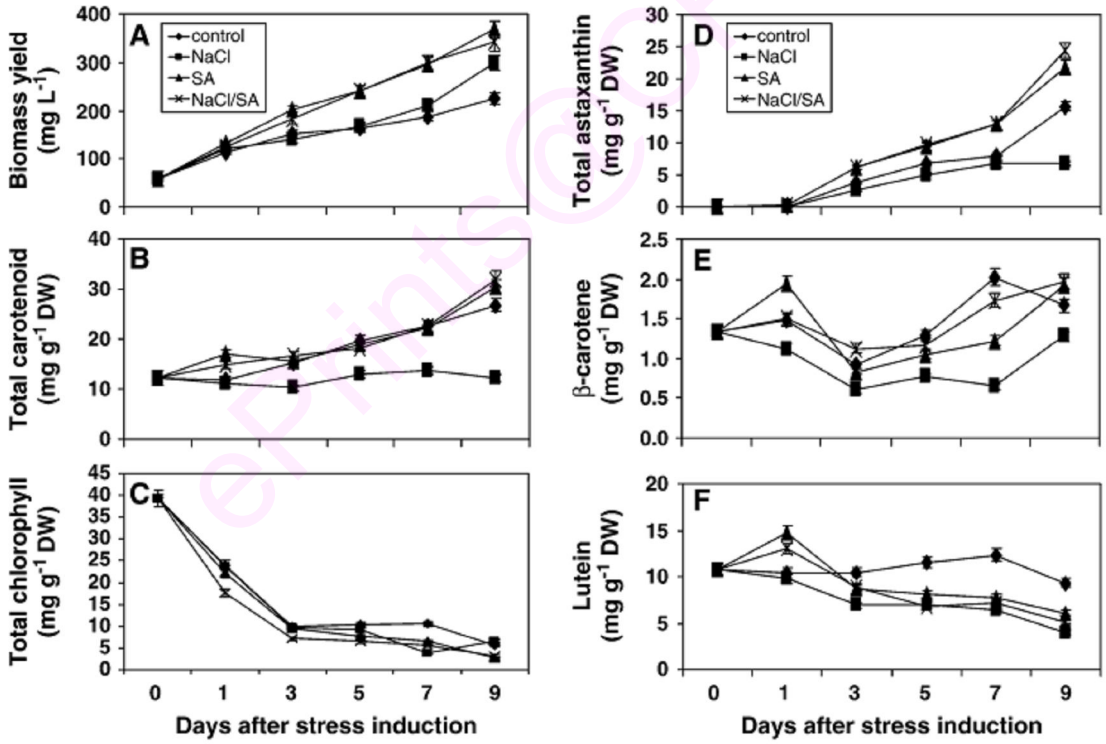


Figure 2.1. Biomass and pigment contents of *H. pluvialis* cultures under stress conditions. After one week of initial growth in autotrophic media, *H. pluvialis* cultures were transferred to nutrient-limiting media and high light (control) along with NaCl, SA, and NaCl/SA addition. Cells were harvested at different periods of incubation and, changes in biomass production (A), total carotenoid (B), total chlorophyll (C), total astaxanthin (D), β-carotene (E), and lutein (F) content were analysed. Values are mean±SD of three independent determinations.

2.3.2. Changes in pigment profile during stress induction

Haematococcus cells collected at different intervals of stress induction were analysed for the changes in content and composition of both carotenoids and chlorophylls. Spectrometric and TLC (Figures 2.2 and 2.3) analyses showed degradation of chlorophyll and accumulation of ketocarotenoids.

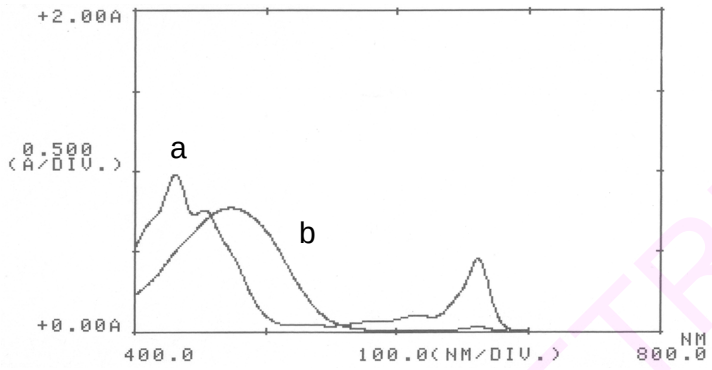


Figure 2.2. Spectrophotometric analysis of carotenoid extracts from *H. pluvialis* (a) - green vegetative and (b) - red cells

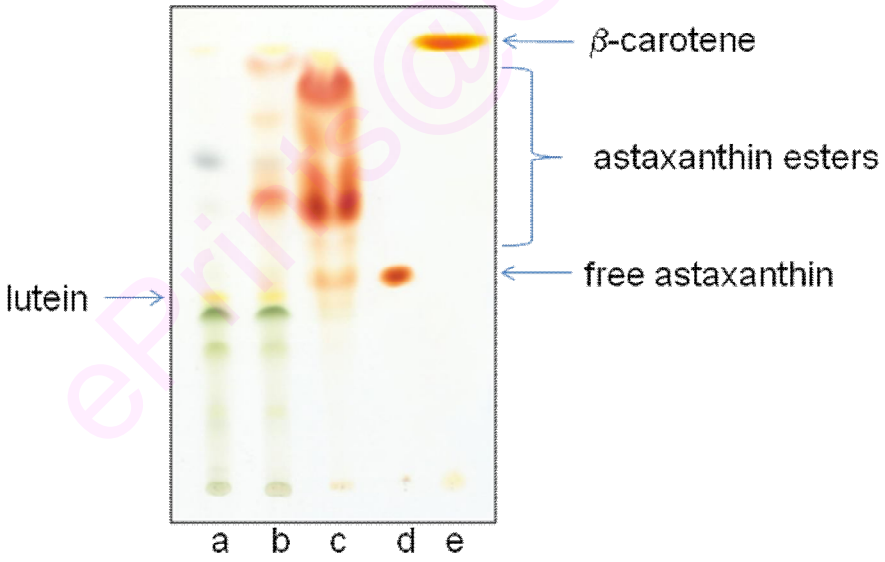


Figure 2.3. TLC analysis of carotenoid extracts from *H. pluvialis* (a) green vegetative, (b) intermediate and (c) encysted red cells, and of standards – (d) free astaxanthin and (e) *β*-carotene

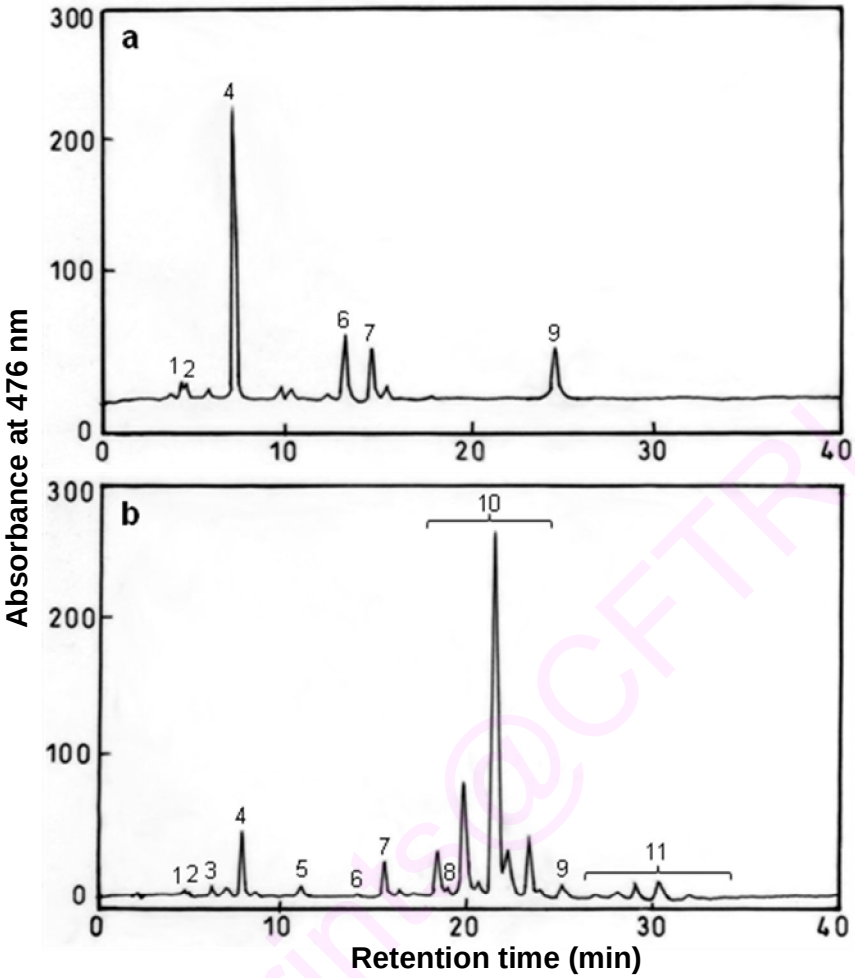


Figure 2.4. HPLC profile of carotenoid extracts from *H. pluvialis* (a) green vegetative cells and (b) red cysts. Peaks were identified as (1) neoxanthin, (2) violaxanthin, (3) free astaxanthin, (4) lutein, (5) canthaxanthin, (6) chlorophyll b, (7) chlorophyll b', (8) chlorophyll a + echinenone, (9) β-carotene, (10) astaxanthin monoesters and (11) astaxanthin diesters.

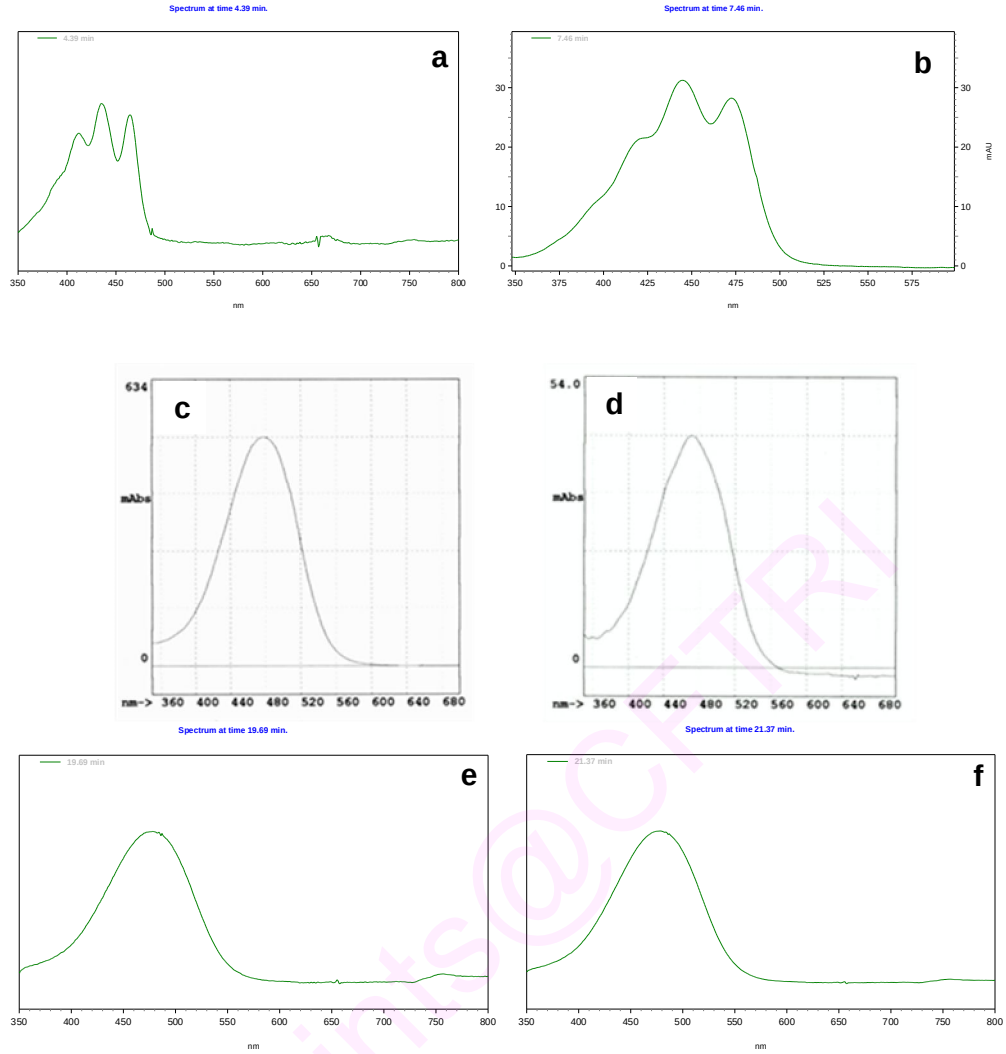


Figure 2.5. Photodiode array spectra of carotenoids (a) neoxanthin, (b) lutein, (c) free Astaxanthin, (d) canthaxanthin and (e and f) astaxanthin ester peaks separated by HPLC

HPLC analyses revealed the occurrence of ketocarotenoids, astaxanthin and its esters, canthaxanthin and Echinenone under stress conditions (Figures 2.4 and 2.5). The initial carotenoid content of green motile cells was 12.1 mg g^{-1} dry weight (DW) (Figure 2.1B) with lutein and β-carotene as major constituents, followed by neoxanthin and violaxanthin occurring in traces, but there were no secondary carotenoids. In our preliminary study, it was revealed that exposure of *H. pluvialis* cells to nutrient-stress and high-light conditions increased astaxanthin production by 3.5-fold over nutrient-sufficient and high-light-exposed cells and 1.5-fold over nutrient-stress and low-light-exposed cells. Upon stress induction, the carotenoid content increased with a concomitant decrease in the chlorophyll content (Figures

2.1B and C). After 9 d of stress, the total chlorophyll content in all the treatments was 90% less than that of green motile cells (Fig. 1C). The addition of NaCl decreased the total carotenoid content, however, addition of SA and NaCl/SA increased the total carotenoid content showing the synergistic effect of SA and NaCl (Figure 2.1B).

In all the treatments, there was an overall decrease in primary carotenoids and especially lutein content under NaCl stress (Figure 2.1F). β-carotene constituted 1.7, 1.3, 1.9, and 2.0 mg g^{-1} DW and lutein constituted 9.3, 3.9, 6.1, and 5.1 mg g^{-1} DW in control, NaCl, SA, and NaCl/SA cultures, respectively (Figures 2.1E and F). Astaxanthin monoester (6.08–22.03 mg g^{-1} DW) and diester (0.7–2.4 mg g^{-1} DW) contents increased while free astaxanthin, canthaxanthin, and echinenone (intermediates in the astaxanthin biosynthetic pathway) were present in traces throughout the experimental period. The fluctuations in their concentration indicated their faster conversion to astaxanthin and its ester forms. At the end of the experimental period, total astaxanthin contents were 15.7, 6.8, 21.8, and 24.5 mg g^{-1} DW produced by control, NaCl-, SA-, and NaCl/SA-added cultures, respectively (Figure 2.1D). Astaxanthin monoester constituted 88–90% while the diester constituted 8–10% of the total astaxanthin under all the stress conditions studied.

2.3.3. Expression analysis of carotenoid genes during stress induction

Transcripts of *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* genes were detected in all stages of stress induction. Both general carotenogenic genes and specific astaxanthin biosynthetic genes were found to be up-regulated upon exposure to various stresses (Figures 2.6A and B). Maximum transcript levels of *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* were found to be 158–277, 5–9, 470–674, 28–40, and 451–673-fold higher, respectively, than green vegetative cells. Exposure to nutrient stress and high light (control) resulted in the early up-regulation of *PSY*, *PDS*, and *LCY* transcripts that reached a maximum on the second day of stress induction. The induction was found to be transient except for *PDS*. As the stress progressed, transcript levels of these genes increased. Maximum levels of *PSY* and *PDS* transcripts were observed on the ninth day when compared with the second day. NaCl addition, delayed the up-regulation of *PSY*, *PDS*, and *LCY* expression, but the transcript levels were similar or higher when compared with other stresses. *PSY* transcripts reached a maximum level on the sixth day while *PDS* and *LCY* transcripts reached their maximum on the ninth day. Addition of SA and NaCl/SA produced an early up-regulation of *PSY*, *PDS*, and *LCY*, which

reached maximum on the ninth day (Figures 2.6A and B). However, their levels were comparatively lower than both control and NaCl-treated cultures.

The increase in transcripts of the astaxanthin biosynthetic genes, *BKT* and *CHY*, in response to nutrient stress and high light intensity was similar to mRNA levels of *LCY*. The addition of NaCl caused delayed up-regulation of *BKT* and *CHY* with their levels reaching a maximum on the third day and the ninth day, respectively. Addition of SA and NaCl/SA produced an early up-regulation and maximum transcripts, compared with control and NaCl treatments. Both *BKT* and *CHY* transcripts reached a maximum on the fourth day in SA-treated cultures. NaCl/SA-treated cultures produced maximum *BKT* transcripts on the third day and *CHY* on the ninth day (Figures 2.6A and B).

2.3.4. Effect of transcriptional and translational inhibitors on carotenoid formation and carotenoid biosynthesis genes expression under nutrient stress conditions

The concentration and composition of carotenoids and the expression of carotenoid genes were studied in stressed cultures treated with transcription and translation inhibitors. Actinomycin D, a transcriptional inhibitor, caused a reduction in total carotenoid and astaxanthin content, although the overall carotenoid composition remained unchanged (Figure 2.7). Cycloheximide, a cytoplasmic translational inhibitor, upon addition to stressed cultures also caused reduction in total carotenoids, but with marked differences in astaxanthin content (Figure 2.7). It caused reduction in astaxanthin content in control cultures and completely inhibited astaxanthin synthesis in other stress cultures. The translational inhibitor at the organellar level, chloramphenicol, exhibited reduction in overall carotenoid and astaxanthin production in all stress cultures except in NaCl-treated cells. Carotenoid inhibition was maximum in NaCl/SA-added cultures. However, chloramphenicol increased the accumulation of lutein (as analysed by HPLC) in all stress conditions except for the NaCl-added cultures where the astaxanthin content was higher. Traces of neoxanthin, violaxanthin, canthaxanthin, and echinenone were detected in all treated cultures.

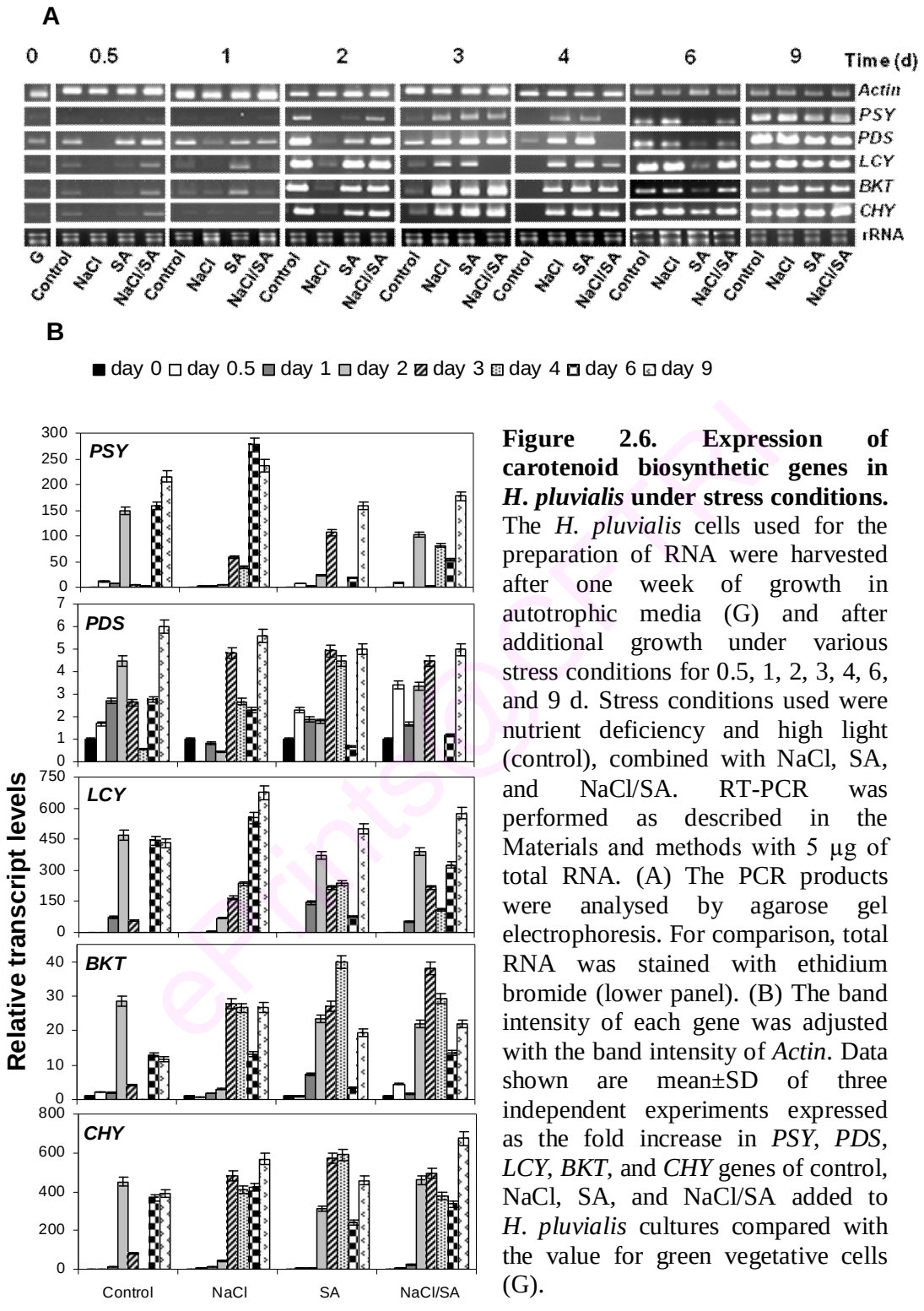


Figure 2.6. Expression of carotenoid biosynthetic genes in *H. pluvialis* under stress conditions. The *H. pluvialis* cells used for the preparation of RNA were harvested after one week of growth in autotrophic media (G) and after additional growth under various stress conditions for 0.5, 1, 2, 3, 4, 6, and 9 d. Stress conditions used were nutrient deficiency and high light (control), combined with NaCl, SA, and NaCl/SA. RT-PCR was performed as described in the Materials and methods with 5 µg of total RNA. (A) The PCR products were analysed by agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (lower panel). (B) The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* genes of control, NaCl, SA, and NaCl/SA added to *H. pluvialis* cultures compared with the value for green vegetative cells (G).

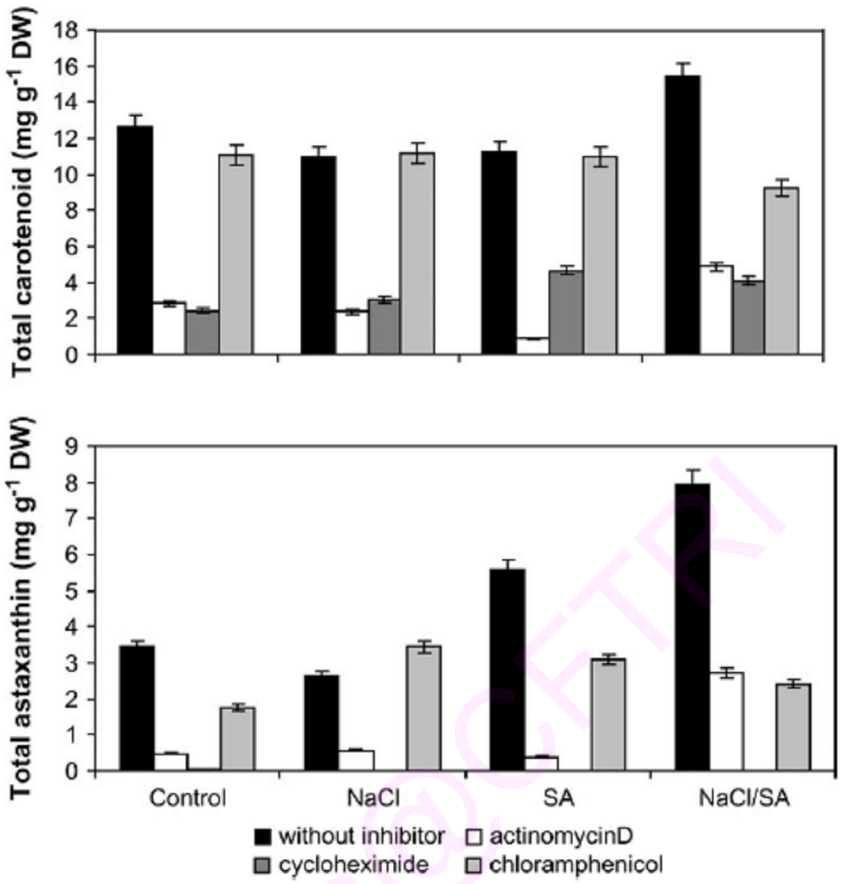


Figure 2.7. Changes in total carotenoid and total astaxanthin contents of *H. pluvialis* cells under the influence of transcriptional and translational inhibitors. Cells grown in autotrophic medium were harvested and transferred to nutrient-limiting medium. Inhibitors, actinomycin D, cycloheximide, and chloramphenicol were added at the time of stress induction. Cultures were harvested 6 d after stress induction, lyophilized, and the carotenoids were analysed. Values are mean±SD of three independent determinations.

As addition of SA and NaCl/SA hastens transcription of carotenoid genes and carotenoid accumulation, the influence of transcriptional and translational inhibitors were studied by adding inhibitors to cultures after 3 d of stress induction. Actinomycin D reduced the total carotenoid and astaxanthin content in all treatments except in the SA-added culture. The extent of reduction was prominent in control and NaCl-treated cultures. However, the decrease in astaxanthin content in the NaCl/SA culture was less (Figure 2.8). The addition of cycloheximide did not reduce total carotenoid in SA-added culture and a considerable reduction was observed in control, NaCl-, and NaCl/SA-added cultures. Total astaxanthin production was completely inhibited in the control and NaCl-added cultures and significantly reduced in SA- and

NaCl/SA-treated cultures (Figure 2.8). This indicated the role of acetate in enhancing total carotenoid and astaxanthin production through post-translational activation. No significant reduction in total carotenoid content was observed when chloramphenicol was added after 3 d of stress induction. However, there was a noticeable decrease in astaxanthin content in control and NaCl/SA-added cultures. It is interesting to note that the decrease in astaxanthin content correlated with the increase in lutein content in the control and NaCl/SA-added cultures indicating the diversion of the carotenoid pathway towards protection of the organism from the inhibitory effect caused by chloramphenicol.

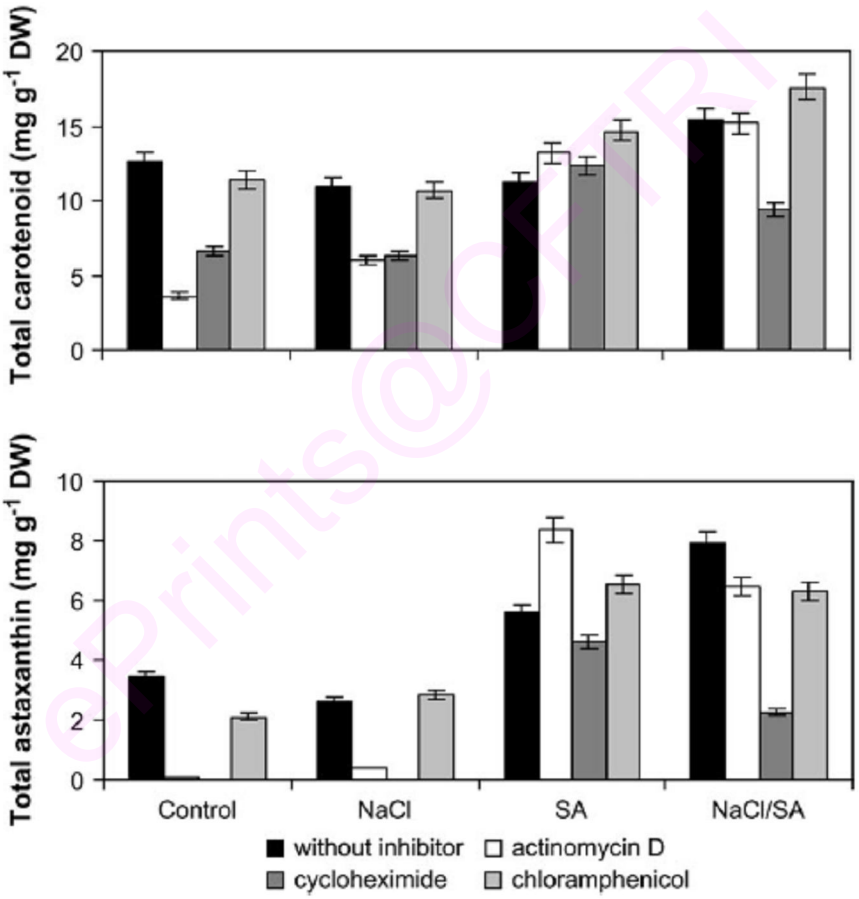


Figure 2.8. Changes in total carotenoid and total astaxanthin contents of *H. pluvialis* cells under the influence of transcriptional and translational inhibitors. Cells grown in autotrophic medium were harvested and transferred to nutrient-limiting medium. Inhibitors were added after 3 d of stress induction and cultures were harvested 6 d after stress induction. Values are mean±SD of three independent determinations.

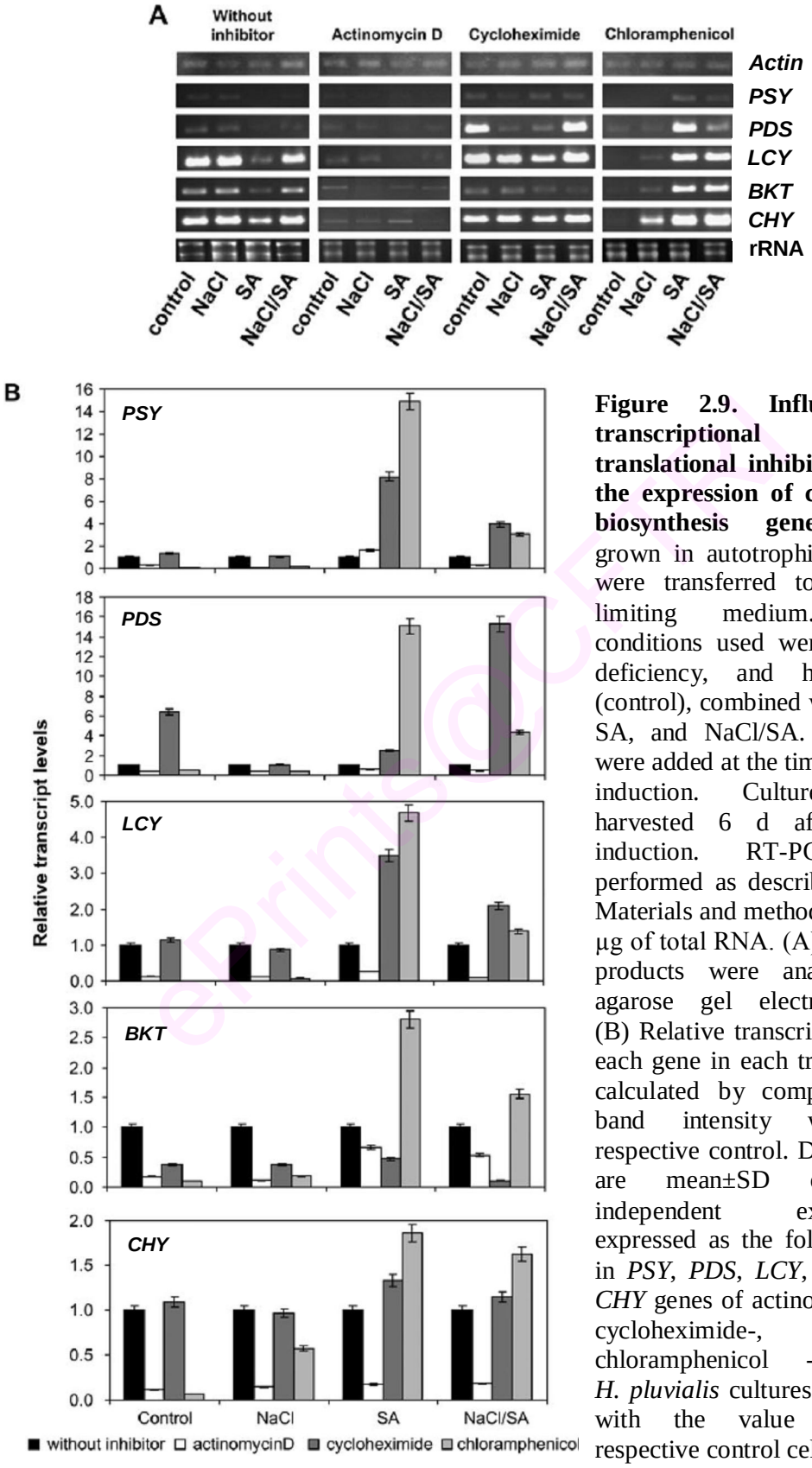


Figure 2.9. Influence of transcriptional and translational inhibitors upon the expression of carotenoid biosynthesis genes. Cells grown in autotrophic medium were transferred to nutrient-limiting medium. Stress conditions used were nutrient deficiency, and high light (control), combined with NaCl, SA, and NaCl/SA. Inhibitors were added at the time of stress induction. Cultures were harvested 6 d after stress induction. RT-PCR was performed as described in the Materials and methods with 0.2 µg of total RNA. (A) The PCR products were analysed by agarose gel electrophoresis. (B) Relative transcript level of each gene in each treatment is calculated by comparing the band intensity with the respective control. Data shown are mean±SD of three independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* genes of actinomycin D-, cycloheximide-, and chloramphenicol - treated *H. pluvialis* cultures compared with the value for the respective control cells.

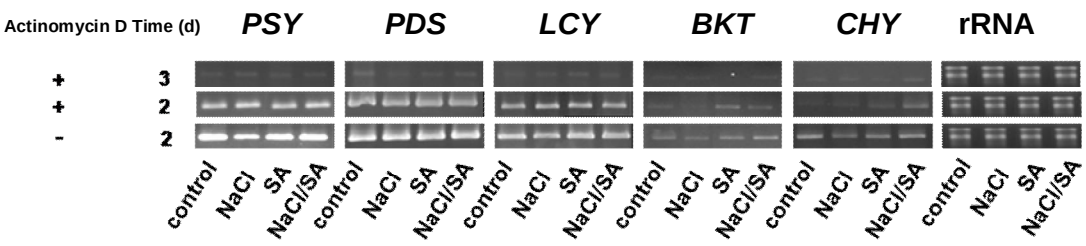


Figure 2.10. Influence of actinomycin D on the expression of carotenoid biosynthesis genes. The inhibitor was added at the time of stress induction and cultures were harvested 2 d or 3 d after stress induction. RT-PCR analysis was performed as described in Figure 2.9.

Actinomycin D reduced expression of all carotenoid biosynthesis genes studied (Figures 2.9A and B). Cycloheximide reduced the expression of only the *BKT* gene under all the conditions studied. There were no noticeable changes in the expression levels of other carotenoid genes in the control and NaCl-treated cultures. However, significant increase in the expression levels of these genes was observed in the presence of SA and NaCl/SA. Chloramphenicol significantly reduced the expression of all genes studied in control and NaCl-added cultures and increased the carotenogenic genes expression in SA- and NaCl/SA-added cultures (Figure 2.9). It is evident from the results that both organellar and cytoplasmic translational inhibitors in the presence of acetate (SA or NaCl/SA) influenced the expression levels of all carotenogenic genes except *BKT* by cycloheximide. To obtain more information on stability of carotenoid mRNAs, transcripts were studied after 2 d and 3 d of actinomycin D addition and stress induction. Carotenogenic gene transcripts exhibited faster degradation under these conditions (Figure 2.10).

2.4. Discussion

In the present work, attempts were made to compare the changes in pigment profile with expression of carotenogenic genes under various stress conditions in *H. pluvialis*. The influence of transcriptional and translational inhibitors on carotenoid accumulation and carotenogenic genes expression was also studied in order to understand the regulation of carotenogenesis. Enhanced cyst formation and astaxanthin accumulation by acetate addition (Figure 2.1D), indicated the role of acetate in increasing C/N ratio thereby enhancing astaxanthin accumulation, as suggested by Kakizono et al. (1992). The addition of NaCl resulted in the reduction of total carotenoid content and astaxanthin content when compared with the control. However, addition of NaCl/SA enhanced the astaxanthin content. Another significant feature observed during stress-induced astaxanthin formation was the decrease in the chlorophyll content (Figure 2.1C), which may be due to nutrient deficiency-induced chlorophyll breakdown (Boussiba et al. 1999).

Generally, secondary carotenoids, namely astaxanthin, canthaxanthin, and echinenone, were detected only after stress induction. However, in the present study, genes for astaxanthin biosynthesis, *BKT* and *CHY*, were found to be expressed at a basal level even in green flagellated cells. During stress, these genes were found to be up-regulated along with other carotenoid genes, namely, *PSY*, *PDS*, and *LCY* (Figures 2.6A and B) and these carotenogenic gene transcripts were detected even in 3-month-old cysts (Vidhyavathi et al. 2007). Differential expression of carotenoid genes during carotenogenesis indicates their probable regulation at different stages of carotenoid accumulation. Among the treatments studied, the addition of SA and NaCl/SA showed the maximum up-regulation of carotenogenic genes. Although NaCl addition favoured up-regulation of carotenogenic genes, expressions were delayed (Figure 2.6). In contrast to this, Steinbrenner and Linden (2001) reported a high level of expression for *PSY* and *CHY* genes, when cells were exposed to high light and NaCl. This difference in expression by NaCl may be due to the difference in mode of cultivation (heterotrophic and autotrophic) and stress induction (nutrient sufficient and deficient). It was also reported that NaCl had a similar effect as SA in inducing the expression of *PSY*, *CHY*, and *BKT*, interpreting that SA initiates a salt stress as does NaCl (Steinbrenner and Linden 2001; Huang et al. 2006a). But the contrasting result

revealed in the present study suggests the involvement of a mechanism other than salt stress in SA-induced transcription of carotenogenic genes. Kovacs et al. (2000) reported that, in *Chlamydobotrys stellata*, the involvement of acetate in the regulation of the redox state of photosystem components thereby affected transcription of nuclear and chloroplast genes. Endo and Asada (1996) also reported plastoquinone reduction stimulated by acetate, thereby affecting photosynthetic activity. These effects of acetate have considerable importance since both general carotenogenic and specific astaxanthin biosynthetic genes were reported to be under photosynthetic redox control (Steinbrenner and Linden 2003). Based on the results from the present study and previous reports, it may be suggested that acetate has a role in enhancing astaxanthin accumulation through photosynthetic redox control.

Increased production of astaxanthin by the addition of SA and NaCl/SA is correlated with early up-regulation and higher expression of astaxanthin biosynthetic genes (Figures 2.1D, 2.6A and B). It may therefore be suggested that enhanced astaxanthin accumulation is correlated with early up-regulation and higher expression of astaxanthin synthesis genes. This is further supported by the absence or less inhibitory effect on carotenoid and astaxanthin accumulation by actinomycin D when added 3 d after SA and NaCl/SA stress induction (Figure 2.8). The absence or reduced inhibition of total carotenoid and astaxanthin production by later addition of cycloheximide in SA and NaCl/SA cultures (Figure 2.8) indicated post-translational activation of carotenoid biosynthesis by acetate. Similar to this, post-translational activation of carotenoid biosynthesis by oxidative stress was reported in acetate-induced cyst cells of *H. pluvialis* (Kobayashi et al. 1993).

The reduction in total carotenoid and astaxanthin contents by transcriptional and cytoplasmic translational inhibitors (Figure 2.7) indicates the regulation of primary and secondary carotenoid formation at both the transcriptional and the cytoplasmic translational levels. Carotenoid genes expression in response to high light and nutrient stress combined with NaCl and SA additions were found to be related to transcriptional activation rather than to the stability of mRNAs as indicated by actinomycin D treatment (Figure 2.10). Inhibition of astaxanthin by cycloheximide substantiates the cytoplasmic translational regulation of secondary carotenogenesis. Cycloheximide addition did not affect the transcription of *PSY*, *PDS*, *LCY*, and *CHY* genes (Figure 2.9). Therefore induction of carotenogenic genes expression in response to stress conditions may be independent of cytoplasmic protein synthesis, at least for

general carotenoid synthesis genes. Similar to this, induction of a higher expression of *PSY* and *CHY* genes of *Haematococcus* by sodium acetate, Fe^{2+} and high light (Steinbrenner and Linden 2001), and mRNA levels of *PSY* gene in corollas of *Cucumis sativus* were shown to be independent of *de novo* protein synthesis (Vishnevetsky et al. 1997). Significant reduction in the expression of *BKT* by cycloheximide showed that regulation of this gene expression differs from other carotenoid genes and induction is dependent on *de novo* protein synthesis in the cytosol. The decrease in *BKT* gene expression by cycloheximide is also reflected in the significant reduction in astaxanthin content. The enhanced expression of some carotenoid genes transcripts upon cycloheximide treatment suggests the involvement of post-transcriptional modifications and stabilization of mRNAs by a translational arrest linked process or by preventing the synthesis of labile nucleases (Price et al. 2004). Although the role of acetate (SA and NaCl/SA) in further enhancing the expression of carotenoid genes (except *BKT*) in the presence of cycloheximide and chloramphenicol is not clear, it suggests a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes. Chloramphenicol reduced astaxanthin production except in NaCl-added cultures (Figure 2.7), which is similar to the report of Brinda et al. (2004). This indicates the involvement of the translation of organellar genes for the enhanced production of secondary carotenoids. This is the first report of its kind where regulation of carotenogenesis, both general and astaxanthin-specific, under the influence of nutrient and other stress conditions has been studied at the expression level and the metabolite level using transcriptional and translational inhibitors. It is evident from this study that acetate plays a crucial role in the enhancement of astaxanthin accumulation. This study will be helpful in understanding the regulation of carotenogenesis and expression of these genes in other organisms.

Chapter III

Regulation of Carotenogenic and Photosynthetic Genes Expression

Summary

Regulation of carotenogenesis in *Haematococcus pluvialis* was studied using various carotenoid and fatty acid synthesis inhibitors under high light in nutrient sufficient (NS/HL) and nutrient deficient (ND/HL) condition. The results showed inhibition of astaxanthin by all inhibitors studied except cerulenin in ND/HL. Total carotenoids were reduced by inhibitors except diphenylamine (DPA) in ND/HL. Lycopene accumulation was observed with nicotine treatment while β-carotene accumulation was observed with DPA. Under NS/HL condition, expression of carotenogenic gene *PSY* was enhanced by all inhibitors studied except DPA while *PDS* expression was reduced by all inhibitors tested except nicotine. Expression of *LCY* was enhanced by inhibitors. Reduced expression of *BKT* and *CHY* were observed under DPA and NF treatment while enhanced expression for *BKT* was observed under nicotine treatment and *CHY* expression under nicotine and cerulenin. ND/HL exposure enhanced carotenogenic genes expression when compared to respective inhibitors of NS/HL in most cases. Reduced expression of *LCY* by nicotine resulted in increased lycopene accumulation while *PSY* and *LCY* by cerulenin resulted in decreased β-carotene and lutein. The results indicate the involvement of other regulatory factors besides transcriptional regulation of carotenogenesis in *H. pluvialis*. Photosynthetic genes, *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* transcripts were up-regulated during stress-induced accumulation of astaxanthin with the maximum of 3.75, 1.66, 1.50, 11.63 and 1.26-fold increase respectively over non-stressed green motile cells. Transcripts of these genes were detected even in cyst cells with the transcript levels of *cabL1818*, *psaB* and *atpB* at reduced level. The nuclear genes *cabL1818* and *lhcbm9* are coding for the chlorophyll *a/b* binding protein L1818 and major light-harvesting complex II m9 protein respectively. The chloroplast encoded *psaB* gene codes for PSI reaction centre protein PsaB. Studies using photosynthetic inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) showed expression of *cabL1818*, *lhcbm9*, *psaB* and *rbcL* were under redox control of plastoquinone pool and *atpB* gene expression may be regulated at cytochrome *b6/f* complex. Expression of all five photosynthetic genes studied was regulated at transcriptional and cytoplasmic translational levels, and their expressions were reduced by norflurazon induced photo-oxidative stress. Acetate modulates the HL induced expression of photosynthetic genes and it depends on redox state of cytochrome *b6/f* complex and cytoplasmic protein synthesis.

3.1. Introduction

In photosynthetic organisms, changes in environmental conditions, such as light quality and quantity, temperature or the availability of nutrients and water, modulates the function of the photosynthetic apparatus. The major proteins of the photosynthetic apparatus in the chloroplast are multi subunit protein complexes that comprise subunits encoded by both the chloroplast and nuclear genomes. The enormous difference in gene copy number between nuclear and chloroplast compartments require a highly coordinated regulation in their expression during development and acclimation of the organisms to environmental cues. This coordination is controlled by the nucleus at many levels (Leon et al. 1998) but also involves signals from the plastids, which influence the expression of nuclear genes for plastid proteins (Pfannschmidt et al. 2001; Leister 2005).

Carotenoids and chlorophylls (Chls) are essential components of photosynthetic apparatus. They are present in functional pigment-binding protein structures embedded in photosynthetic membranes. β-carotene is more abundant in the reaction centres of photosystems I and II, and xanthophylls are preferentially distributed in the light-harvesting complexes (Demmig-Adams et al. 1996). Carotenoids serve at least two important functions in photosynthesis. They participate in the light-harvesting process and protect the photosynthetic apparatus from photo-oxidative damage (Bartley and Scolnik 1995). They are essential for the efficient quenching of Chl triplets formed by an excess of light and they dissipate excess light energy through non-photochemical quenching (NPQ) of Chl fluorescence (Muller et al. 2001). Carotenoid biosynthesis takes place in the plastid, but all known enzymes in the pathway are nuclear-encoded and post-translationally imported into the organelle (Lichtenthaler 1999). Microalgal cells possess carotenoids normally found in the chloroplast of higher plants, namely neoxanthin, violaxanthin, lutein, zeaxanthin and β-carotene, also referred to as primary carotenoids. The secondary carotenoids almost exclusively accumulate under stress conditions (e.g. nutrient starvation, salinity, high temperature etc.) and this process is species specific. The physiological function of secondary carotenoid remains to be clarified (Boussiba 2000). However, it is generally believed that they function as passive photoprotectants (i.e. as a filter)

reducing the amount of light which can reach the light-harvesting pigment complex of PS II (Hagen et al. 1994).

Astaxanthin accumulation in *H. pluvialis* is accompanied by loss of flagella, formation of cysts, and reduction in photosynthetic activity etc. The decline in photosynthesis is attributed to the damage to the PS II complex (Zlotnik et al. 1993), lack of cytochrome *f* and the expected absence of linear electron flow from PSII to PSI and the decrease of some of the PSI and PSII components (Tan et al. 1995). Under stress conditions, astaxanthin is accumulated in extra-plastidic lipid vesicles (Grünewald et al. 2001) predominantly in an ester form and astaxanthin accumulation is accompanied by triacylglycerol accumulation (Zhekisheva et al. 2005). General carotenoid and astaxanthin specific genes are up-regulated during stress-induced secondary carotenoid formation and these genes are regulated at transcriptional, post-transcriptional and translational levels (Sun et al. 1998; Grünewald et al. 2000; Steinbrenner and Linden 2001; Vidhyavathi et al. 2008), and they are under the control of photosynthetic redox status (Steinbrenner and Linden 2003). Apart from cultivation parameters, understanding the molecular basis of stress-induced astaxanthin accumulation in *H. pluvialis* will be useful for the optimization of astaxanthin formation.

In photosynthetic organisms, the carotenoid levels reflect a steady state governed by biosynthesis and degradation. The role of pigments in the regulation of carotenoid pathway, either at the level of gene expression or of allosteric feedback control on the enzymatic steps, still remains to be elucidated. Earlier studies on expression of carotenogenic genes under the influence of inhibitors of carotenogenesis and fatty acid synthesis in plant systems are mostly limited to *PSY* or *PDS*. It is demonstrated that carotenoid content and/or composition could influence the expression of some carotenogenic genes. For example, in sunflower phytoene accumulation decreases the transcript levels of the *PSY* gene (Campisi et al. 2006). It has been found that in *Arabidopsis thaliana*, *PDS* expression is independent of leaf pigment content (Wetzel and Rodermel 1998). In daffodil flowers inhibition of lycopene cyclase leads to up-regulation of *PSY*, *PDS* and *LCY* (Al-Babili et al. 1999). In *H. pluvialis* also, it has been found that accumulation of canthaxanthin and/or echinenone depresses the enzyme β-carotene oxygenase activity through a feedback mechanism. Moreover active synthesis of fatty acid is required for astaxanthin

synthesis (Schoefs et al. 2001), which raised a question whether the fatty acid synthesis is required for the esterification of the astaxanthin molecules and/or for the formation of the astaxanthin droplets. Recent findings showed triacylglycerol accumulation is not tightly coupled with astaxanthin accumulation but accumulation of certain amount of triacylglycerol is a prerequisite for the initiation of fatty acid-esterified astaxanthin accumulation in lipid globules (Zhekisheva et al. 2005). The application of various inhibitors blocking specific reactions in the carotenoid biosynthesis pathway would either increase or decrease the pool sizes of particular carotenoids, which in turn would be helpful in studying biochemical and molecular mechanisms behind carotenogenesis. In *Haematococcus*, carotenoid synthesis inhibitors were used to elucidate the pathway of astaxanthin production, and these inhibitors can also be used to analyze the effect of carotenoids on carotenoid genes expression. Diphenylamine (DPA) inhibits β-carotene ketolase and nicotine inhibits lycopene cyclase (Harker and Young 1995). Bleaching herbicide norflurazon (NF) is an inhibitor of phytoene desaturase, which is the first enzyme responsible for introduction of the conjugated double bonds (Boger and Sandmann 1983). Fatty acid synthesis inhibitor cerulenin inhibits β-ketoacyl-(acyl carrier protein) synthase I (Walsh et al. 1990).

Expression of photosynthetic genes and their regulation has been studied in many organisms including *Arabidopsis, Chlamydomonas* and *Dunaliella*. Sequences of few of photosynthetic genes of *Haematococcus* are available in NCBI GenBank. Among these, genes which are having important photosynthetic function are selected for the present study. Unfortunately, to our knowledge, genes of photosystem II (PSII) of *Haematococcus* are not available in database. Our previous study had shown, *H. pluvialis* carotenoid synthesis genes are up-regulated during stress induction by high light and nutrient deficiency and are regulated at transcriptional, post-transcriptional and translational levels (Vidhyavathi et al. 2008). It has been reported that *H. pluvialis* carotenoid biosynthetic genes are regulated at photosynthetic redox control (Steinbrenner and Linden 2003).

Hence the present study has been undertaken to study the regulation of carotenogenic and photosynthetic genes expression. Expression of carotenogenic genes such as *PSY, PDS, LCY, BKT* and *CHY* were studied using inhibitors of carotenoid and fatty acid synthesis. Expression of photosynthetic genes such as *cabL1818, lhcbm9, rbcL, psaB* and *atpB* of *H. pluvialis* and their regulation using

inhibitors of photosynthetic electron transport, transcription, translation as well as carotenoid synthesis under stress conditions were studied.

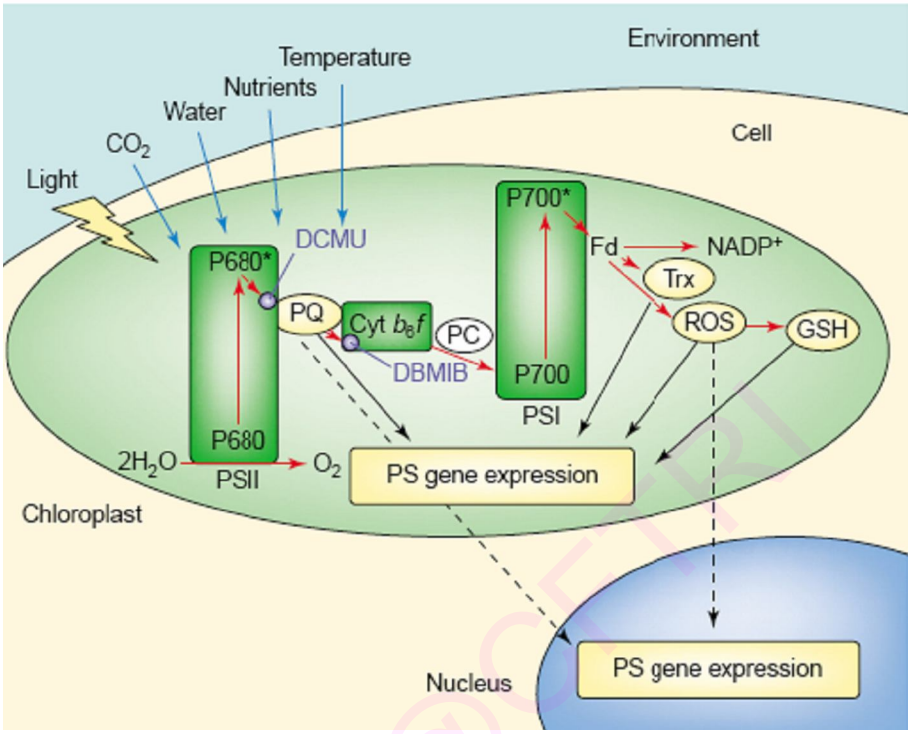


Figure 3.1. Factors in photosynthetic redox chemistry that influence photosynthesis gene expression. The electron transport chain of a chloroplast is drawn schematically according to the Hill–Bendall Z scheme and the electron flow is represented by red arrows (Adopted from Pfannschmidt 2003).

The nuclear genes *cabL1818* and *lhcbm9* are coding for the chlorophyll *a/b* binding protein L1818 and major light-harvesting complex II m9 protein respectively. The chloroplast encoded *psaB* gene codes for PSI reaction centre protein PsaB. The chloroplast genes *rbcL* and *atpB* are coding for large subunit of Ribulose bisphosphate carboxylase oxygenase (Rubisco) and ATP synthase β-subunit respectively. To study the photosynthetic redox control of photosynthetic genes expression, photosynthetic electron transport (PET) inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) were used. DCMU inhibits the oxidation of the primary electron acceptor of PSII, Q_A and consequently the reduction of the secondary acceptor, plastoquinone, and cytochrome *b6/f* cannot be further reduced by PSII (Figure 3.1; Crane 1977). DBMIB inhibits electron transport from plastoquinone (PQ) to the cytochrome *b6/f* complex and it also prevents PQ reoxidation (Trebst 1980).

3.2. Materials and Methods

3.2.1. Algal culture, growth and stress conditions

Haematococcus pluvialis motile vegetative cells are grown in modified BBM medium as described in section 2.2.3 and harvested by centrifugation at 3500 × *g* for 5 min and resuspended in fresh media (complete and nutrient deficient - containing 1/10 of the nitrogen and phosphorus of the original medium). The initial cell concentration was adjusted to 15×10^4 cells ml^{-1}. The cultures were subjected to the following stress conditions (i) high light intensity (HL), (ii) nutrient deficiency and high light (ND/HL), (iii) sodium acetate 4.4 mM and high light (SA/HL). High light intensity refers continuous light intensity of 97 µmol m^{-2} s^{-1}. Cyst cells used for comparative study were produced by exposing green motile vegetative cells to HL for three months.

3.2.2. Inhibitors

DPA (Sigma) stock was prepared in 70% basic ethanol (pH 9.0) and used at final concentration of 45 µM. (-)-Nicotine (Fluka) was added to the cultures at a final concentration of 9.8 mM. NF (AccuStandard, USA) stock was prepared in methanol and added to cultures at a final concentration of 8.5 µM. Cerulenin (Sigma) stock prepared in methanol was added to cultures at a final concentration of 3.4 µM. The photosynthetic electron transport inhibitors, DCMU (sigma) and DBMIB (sigma) were prepared as stock solutions in DMSO and methanol respectively and added to cultures at a final concentration of 20 µM and 1 µM respectively. DBMIB was re-supplied every 5-6 h because of its photo-labile nature (Steinbrenner and Linden 2003). The transcriptional inhibitor actinomycin D (SRL, Mumbai, India), the cytoplasmic translational inhibitor, cycloheximide (Sigma-Aldrich, Bangalore, India), and the organellar translational inhibitor, chloramphenicol (HiMedia, Mumbai, India) were added at concentrations of 10 µg ml^{-1}, 300 ng ml^{-1}, and 50 µg ml^{-1}, respectively. After the addition of inhibitors cultures were exposed to continuous high light intensity of 97 µmol m^{-2} s^{-1} for two days for secondary carotenoid induction.

3.2.3. Growth and pigment analyses

Algal cell number was determined by counting algal cells using Neubauer haemacytometer. Known volume of culture was centrifuged at 3000 × *g* for 10 min and dry weight of the biomass was estimated as described in section 2.2.5.2. An aliquot of culture was harvested and freeze dried. Pigment extraction and analyses were carried out as per the procedure given in section 2.2.6.

3.2.4. Chlorophyll fluorescence measurements

Cell suspensions were normalized to about 3 µg chlorophyll ml^{-1} and dark adapted for 30 min. The Chlorophyll fluorescence measurements were carried out using Shimadzu RF-5301PC spectrofluorophotometer (excitation, 450 nm; emission, 690 nm). The initial (F0) and maximum (Fm) fluorescence were measured before and after adding DCMU to a final concentration of 10 µM. Maximum photochemical quantum yield of PSII was determined as the ratio of Fv/Fm (Fv=Fm–F0) (van Kooten and Snel 1990).

3.2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction and removal of genomic DNA were done as per the procedure given in section 2.2.7. First strand cDNA synthesis was performed in a 20-µl reaction volume using 1.5 µg of total RNA, oligo dT_{18} and M-MLV reverse transcriptase (Ambion) according to manufacturer's protocol. The reaction was allowed to proceed for 60 min at 42°C before being terminated by treatment at 92°C for 10 min. The primers used for amplification of *PSY*, *PDS*, *LCY*, *BKT*, *CHY* and *Actin* and amplification conditions are described in section 2.2.7. The primers used for amplification of photosynthetic genes *cabL1818*, *lhcbm9*, *rbcL*, *psaB*, *atpB* and amplification conditions are listed in Table 3.1. Following the separation of the PCR products on ethidium bromide-stained 1.4% agarose gels, the bands were quantified. Each band was normalized against the intensity obtained with the same cDNA using the *Actin*-specific primers. For calculating the transcript abundance under stress conditions, the transcript levels of each gene in green motile cells were taken for comparison, while under inhibitor-added conditions, transcripts of the respective control (without inhibitors) were used for comparison.

Table 3.1. Gene-specific primers and annealing temperatures used for RT–PCR

Primer	Sequence 5'-3'	Product size (bp)	Annealing temperature °C	GenBank ID
cabL1818-F	CCTGAGGCATTGAAGGACAT	458	60	AY786531
cabL1818-R	CCCTCACAGCCAGCTTCTAC			
lhcbm9- F	GTCACCCCTGAGCTGCTG	523	60	AY786530
lhcbm9-R	AACTCGAAGCCGCAAATG			
psaB-F	ATACCGGAATCACGAGGTGT	593	60	AB084365
psaB-R	CGAATCCAGAAAATAGCACCA			
rbcL-F	ATGTGGTGCAGCAGTAGCAG	435	60	AB084336
rbcL-R	AGACCACCGCGTAAACATTC			
atpB-F	ACCTATCCACCGTCAAGCAC	617	60	AB084325
atpB-R	CTGTAAGGTCATCGGCTGGT			

3.2.6. Experimental design and data analysis

Each experiment was repeated three times with at least three replications. Statistical analyses were carried out as described in section 2.2.8.

3.3. Results

3.3.1. Analysis of carotenoids biosynthetic genes expression

3.3.1.1. Changes in pigments under the influence of carotenoid and fatty acid synthesis inhibitors

Green motile *Haematococcus* culture which was used for stress induction and inhibitors treatment contained 37.25±1.25 mg g^{-1} of total chlorophyll or Chl (a+b), 37.65±1.30 mg g^{-1} of total carotenoid with lutein (34.3±1.12 mg g^{-1}), β-carotene (3.35±1.10 mg g^{-1}) and traces of neoxanthin and violaxanthin, and no astaxanthin. After the cells were exposed to high light in nutrient sufficient condition (NS/HL) at 97 µmol m^{-2} s^{-1} for 48 h, Chl (a+b) and carotenoid contents decreased to 31.07±0.95 and 34.47±0.99 mg g^{-1} respectively, whereas astaxanthin, β-carotene and lutein contents were 0.02±0.001, 1.99±0.01 and 32.47±0.98 mg g^{-1} respectively.

Table 3.2. Pigment contents (mg g^{-1} biomass) under the influence of carotenoid and fatty acid synthesis inhibitors

	Total carotenoids	Astaxanthin	β-carotene	Lutein	Lycopene	Chl (a+b)
Nutrient sufficient and High light (NS/HL)						
Control	34.47 ^{a}	0.02 ^{cd}	1.99 ^{bc}	32.47 ^{a}	0 ^{c}	31.07 ^{a}
DPA	26.52 ^{b}	0 ^{e}	2.91 ^{ab}	23.60 ^{b}	0 ^{c}	25.68 ^{b}
Nicotine	18.36 ^{c}	0 ^{e}	1.14 ^{cd}	16.78 ^{cd}	0.44 ^{ab}	19.54 ^{c}
NF	20.22 ^{c}	0 ^{e}	1.48 ^{c}	18.74 ^{c}	0 ^{c}	24.12 ^{b}
Cerulenin	27.30 ^{b}	0 ^{e}	2.72 ^{ab}	24.58 ^{b}	0 ^{c}	22.67 ^{bc}
Nutrient deficient and High light (ND/HL)						
Control	26.54 ^{b}	0.03 ^{cd}	2.16 ^{b}	24.36 ^{b}	0 ^{c}	22.73 ^{bc}
DPA	28.20 ^{b}	0.005 ^{d}	3.11 ^{a}	25.08 ^{b}	0 ^{c}	25.76 ^{b}
Nicotine	9.99 ^{d}	0.01 ^{d}	0.43 ^{d}	8.94 ^{de}	0.61 ^{a}	10.90 ^{de}
NF	16.35 ^{d}	0 ^{e}	1.00 ^{cd}	15.35 ^{bc}	0 ^{c}	22.37 ^{bc}
Cerulenin	16.22 ^{d}	0.10 ^{a}	1.41 ^{c}	14.71 ^{cd}	0 ^{c}	15.26 ^{d}

Each value represents the mean of two separate experiments each with three replicates. Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Neoxanthin, violaxanthin, echinenone and canthaxanthin were present in trace amounts in cells exposed to NS/HL. Treatments with inhibitors DPA, nicotine, NF and cerulenin reduced the total carotenoid contents to 0.70, 0.53, 0.59 and 0.79-fold

respectively of control – HL exposed cells (Table 3.2). Similarly Chl (a+b) contents also showed a decline in inhibitor treated cells (Table 3.2). The inhibitor treated cultures resulted in a reduction in lutein content to 0.73, 0.52, 0.58 and 0.76-fold of control cells in DPA, nicotine, NF and cerulenin treatments respectively. Both DPA and cerulenin enhanced β-carotene accumulation to 1.46 and 1.37-fold (Table 3.2). Neoxanthin and violaxanthin were present in trace amounts in all treated cultures. The accumulation of astaxanthin was inhibited completely in treatments with all inhibitors studied. Lycopene accumulation was observed only with nicotine treatment (0.44 mg g^{-1}). The accumulation of lycopene observed in the present study, by nicotine treatment, was confirmed by exposure of inhibitor treated cultures to HL for another 2 days which resulted in enhanced accumulation of lycopene (1.45 mg g^{-1}).

Under nutrient deficient high light (ND/HL) conditions, decrease in total carotenoids and Chl (a+b) contents were observed when compared to NS (Table 3.2). This decrease in total carotenoids could be attributed to the rapid degradation of lutein and β-carotene during initial stages of secondary carotenoid synthesis in ND/HL. When individual carotenoids were analysed, higher astaxanthin and β-carotene accumulation and reduction in lutein were observed under ND/HL condition (Table 3.2). This indicated the diversion of carotenoid biosynthetic pathway more towards secondary carotenoid synthesis. Under the influence of inhibitors, reduction in total carotenoids and Chls was observed for all inhibitors studied but for DPA, which showed accumulation of β-carotene and lutein compared to control – ND/HL cells. This substantiates the effect of DPA on *BKT* (astaxanthin specific enzyme) and in turn secondary carotenogenesis but not on general carotenoid synthesis. Drastic reduction in lutein content by nicotine, NF and cerulenin treatments reflected in decreased total carotenoid content. Inhibition of astaxanthin accumulation was observed under DPA, nicotine and NF with NF having complete inhibition on astaxanthin accumulation. On the contrary, increased astaxanthin accumulation was observed under cerulenin treatment (Table 3.2).The accumulation of lycopene found in nicotine treated cells indicates the inhibition of lycopene cyclase.

3.3.1.2. Changes in carotenoid synthesis genes expression

To study further the effect of inhibitors on carotenogenic genes expression, RNA was extracted from inhibitor treated samples and transcript levels were compared by RT-PCR. The RT-PCR method was chosen considering the low abundance of many

carotenoid biosynthetic gene steady state mRNAs (Giuliano et al. 1993). Genes selected for RT-PCR in this study are *PSY*, *PDS*, *LCY*, *BKT* and *CHY* (Figure 3.2).

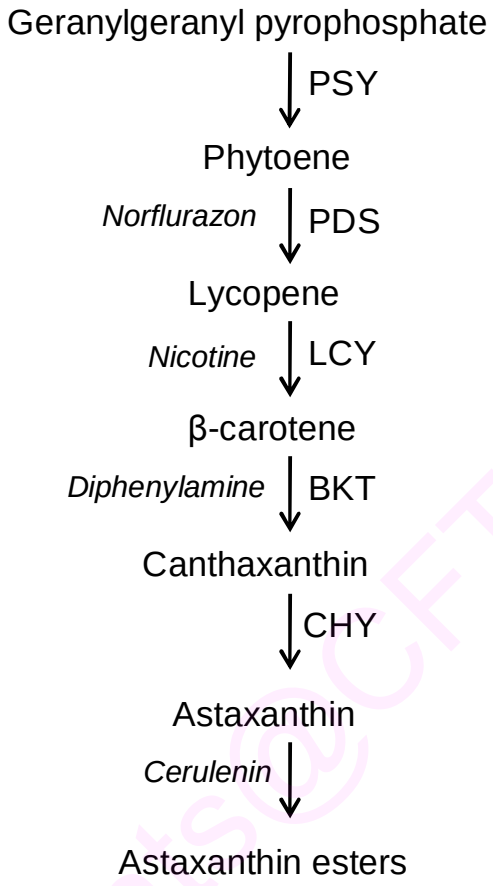


Figure 3.2. Simplified astaxanthin biosynthetic pathway showing enzymes of carotenoid biosynthesis and sites of inhibitors action. Enzyme designation is according to the corresponding gene: PSY, phytoene synthase; PDS, phytoene desaturase; LCY, lycopene cyclase; BKT, β-carotene ketolase; CHY, β-carotene hydroxylase.

Expression of these carotenogenic genes were studied on the 2nd day since all these genes have shown significant up-regulation on 2nd day (Figure 2.6). Figures 3.3 and 3.4 shows that the expression of *PSY* was enhanced by inhibitor treatments but for DPA, which reduces this gene expression to 0.22-fold of control. The increase in expression of *PSY* was observed to be 4.79, 1.97 and 3.36-fold respectively for nicotine, NF and cerulenin treated cultures. The expression of *PDS* was reduced by inhibitors except nicotine, where the expression of this gene was increased to 1.31-fold.

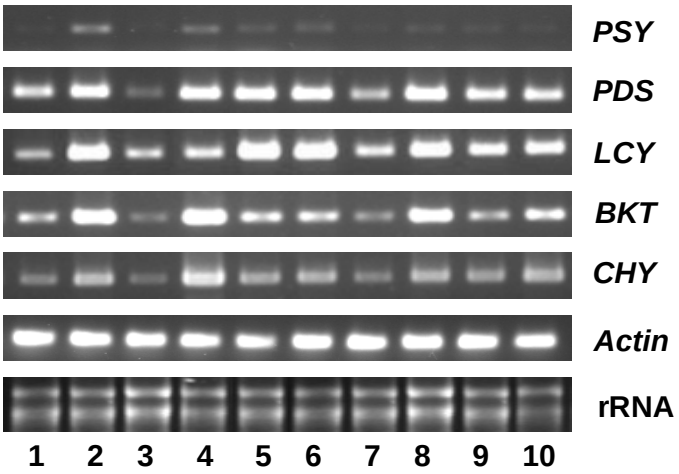


Figure 3.3. Influence of carotenoid and fatty acid synthesis inhibitors on carotenogenic genes expression using RT-PCR. RT-PCR was performed using RNAs from cells grown in NS (lanes 1, 3, 5, 7 and 9) or ND (lanes 2, 4, 6, 8 and 10) media without inhibitors (lanes 1 and 2), in the presence of DPA (lanes 3 and 4), nicotine (lanes 5 and 6), NF (lanes 7 and 8) and cerulenin (lanes 9 and 10). PCR amplified products were separated on 1.5% agarose gel. For comparison, total RNA was stained with ethidium bromide (lower panel).

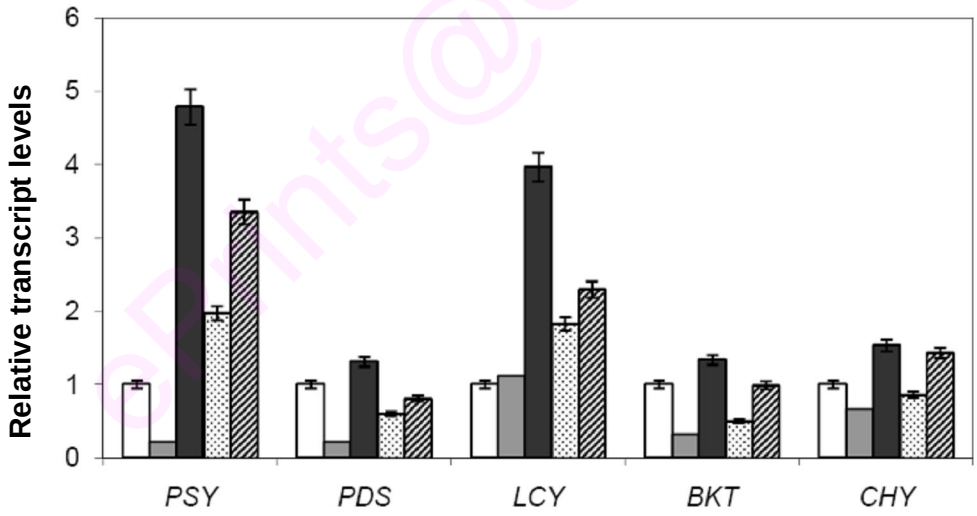


Figure 3.4. Influence of carotenoid and fatty acid synthesis inhibitors upon carotenogenic genes expression in NS condition using RT-PCR. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT* and *CHY* genes of inhibitor added HL exposed cells compared with HL alone.

The reduction in expression of *PDS* by DPA, NF and cerulenin treatments were found to be 0.21, 0.60 and 0.81-fold respectively. DPA, nicotine, NF and cerulenin treated cultures exhibited enhanced *LCY* gene expression of 1.12, 3.96, 1.83

and 2.30-fold respectively. *BKT* gene expression was reduced by DPA and NF to 0.32 and 0.50-fold respectively, while nicotine treatment increased the expression to 1.34-fold and cerulenin treatment did not change the expression. The *CHY* gene expression was reduced by DPA and NF to 0.66 and 0.86-fold respectively whereas increased expression was observed in the presence of nicotine and cerulenin (1.53 and 1.43).

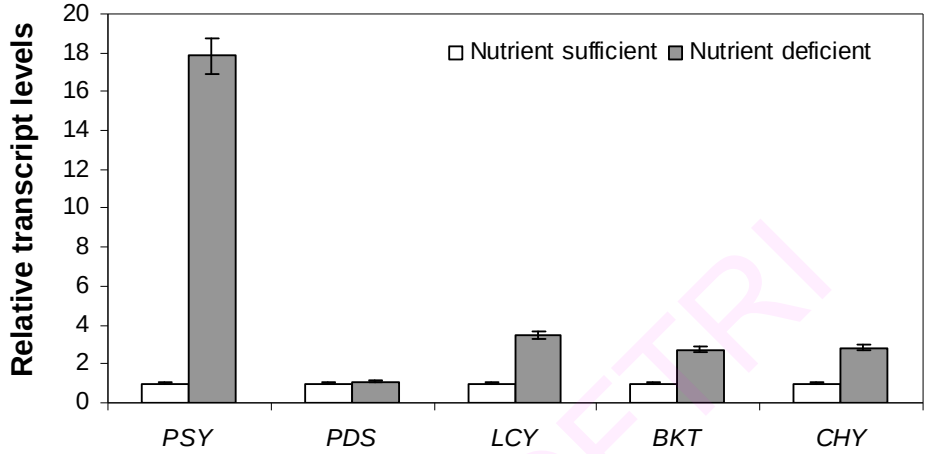


Figure 3.5. Effect of ND upon carotenogenic genes expression in comparison with NS using RT-PCR. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT* and *CHY* genes of ND/HL exposed cells compared with NS/HL.

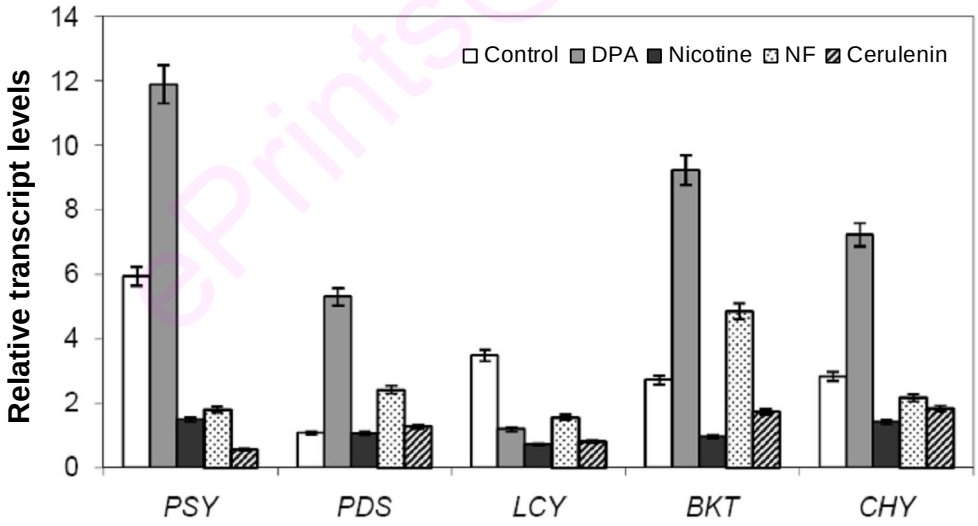


Figure 3.6. Influence of carotenoid and fatty acid synthesis inhibitors upon carotenogenic genes using RT-PCR in ND condition in comparison with NS. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT* and *CHY* genes of inhibitor added ND/HL exposed cells in comparison with band intensities of each gene of respective inhibitor added NS/HL exposed cells. For clarity, relative intensities of *PSY* of control and DPA treated cultures were reduced by 3- and 5-times respectively.

Expression of carotenogenic genes under ND condition compared to NS condition under high light revealed that expression of *PSY*, *PDS*, *LCY*, *BKT* and *CHY* were 17.84, 1.09, 3.49, 2.73 and 2.84-fold higher respectively (Figure 3.5). All the inhibitors showed enhanced expression of carotenogenic genes under ND compared to respective inhibitor condition under NS except *LCY* and *BKT* of nicotine and *PSY* and *LCY* of cerulenin treated cells (Figure 3.6).

3.3.2. Analysis of photosynthetic genes expression

3.3.2.1. Changes in pigment profile and chlorophyll fluorescence under stress conditions

Pigment and Chl fluorescence of cells exposed to HL, ND/HL and cyst cells were measured. Green non-stressed cells contain 37.25 mg g^{-1} of Chl (a+b) with a Chl a/b ratio of 2.06. Total carotenoid content was 37.65 mg g^{-1} with lutein (34.3 mg g^{-1}) and β-carotene (3.35 mg g^{-1}) constituting as major carotenoids followed by neoxanthin and violaxanthin occurring in traces with no astaxanthin.

Table 3.3. Pigment (mg g^{-1}) and chlorophyll fluorescence characteristics under stress conditions

Treatments	Chl (a+b)	Chl a/b ratio	Total carotenoid	Astaxanthin	Lutein	β-carotene	Fv/Fm
Green cells	37.25 ^{a}	2.06 ^{a}	37.65 ^{a}	0 ^{d}	34.30 ^{a}	3.35 ^{a}	0.83 ^{a}
HL 2d	31.07 ^{ab}	2.15 ^{a}	34.47 ^{ab}	0.02 ^{c}	32.47 ^{a}	1.99 ^{b}	0.75 ^{ab}
ND/HL 2d	22.73 ^{b}	2.09 ^{a}	26.54 ^{cd}	0.03 ^{c}	24.36 ^{ab}	2.16 ^{b}	0.63 ^{bc}
Cysts	2.09 ^{cd}	1.27 ^{c}	32.39 ^{b}	31.91 ^{a}	0.31 ^{c}	0.17 ^{cd}	0.49 ^{cd}

The green motile *H. pluvialis* cells were exposed to HL for 2 days (HL 2d), 3 months (cysts) or ND/HL for 2 days (ND/HL 2d). Each value represents the mean of two separate experiments each with three replicates. Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

After the exposure of cells to HL and ND/HL for two days, Chl (a+b) was decreased to 31.07 and 22.73 mg g^{-1} and total carotenoid to 34.47 and 26.54 mg g^{-1} respectively, while Chl a/b ratio has been increased to 2.15 and 2.09 respectively (Table 3.3). Though stress conditions reduced the total carotenoid content, it increased the proportion of astaxanthin among total carotenoids (Table 3.3). Astaxanthin, β-carotene and lutein contents were 0.02-0.03, 1.99-2.16 and 32.47-24.36 mg g^{-1} respectively in HL and ND/HL exposed cells. Cyst cells were found to possess very

low level of Chl (2.09 mg g^{-1}) with a low Chl a/b ratio of 1.27 (Table 3.3). Total carotenoid content of cyst cells was 32.39 mg g^{-1} with astaxanthin, β-carotene and lutein constituting 31.91, 0.17 and 0.31 mg g^{-1} respectively. Traces of violaxanthin, neoxanthin, echinenone and canthaxanthin were present in HL, ND/HL and cyst cells. Maximum quantum yield of PSII (Fv/Fm) of green non-stressed cells was 0.83. Under stress conditions Fv/Fm was found to be reduced to 90, 76, and 59% of green non-stressed cells in HL, ND/HL and cyst cells respectively (Table 3.3).

3.3.2.2. Differential expression of photosynthetic genes under stress conditions

Transcript levels of photosynthetic genes at different stages of stress induction were studied and compared with the transcript levels of respective genes in green non-stressed cells. Exposure of cells to high light intensity (HL) for 2 days increased the transcript levels of *cabL1818*, *lhcbm9* and *rbcL* to 1.96, 1.66 and 2.15-fold respectively and further exposure of cells to HL increased the transcript levels of *psaB* and *atpB* (Figure 3.7).

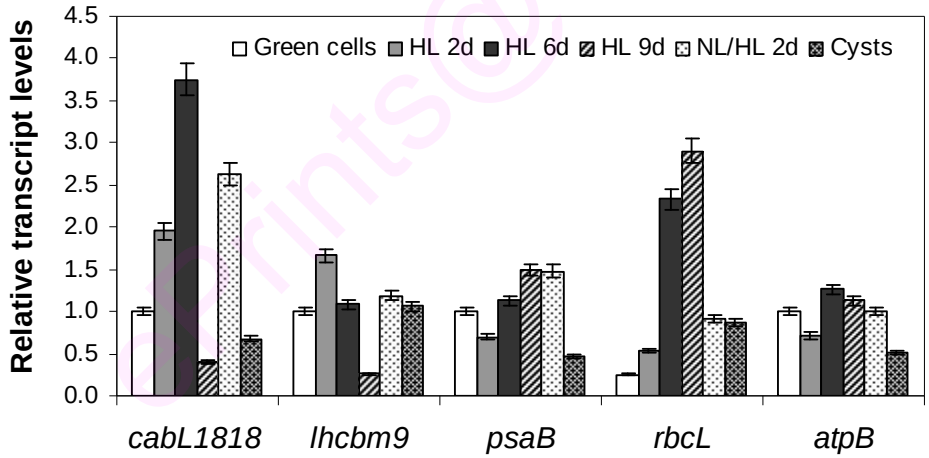


Figure 3.7. Expression of photosynthetic genes in *H. pluvialis* under stress conditions. The green motile *H. pluvialis* cells were exposed to HL for 2 days (HL 2d), 6 days (HL 6d), 9 days (HL 9d), 3 months (cysts) or ND/HL for 2 days (ND/HL 2d). RNA extraction and RT-PCR were performed as described in the materials and methods with 1.5 µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* genes of HL, ND/HL exposed and cyst cells compared with the value for green vegetative cells.

HL induced expression was most prominent for *rbcL* among the genes tested. Maximum transcript levels for *lhcbm9* was found to be on 2nd day (1.66-fold) while *psaB* and *rbcL* produced maximum transcripts on 9th day (1.50 and 11.63-fold) and *cabL1818* and *atpB* on 6th day (3.75 and 1.26-fold). Nutrient deficiency along with high light intensity (ND/HL) for 2 days increased the transcript levels of photosynthetic genes studied except *rbcL* and the up-regulation of *atpB* is not significant compared to green motile cells. Transcripts of photosynthetic genes studied were found to be present in 3 months old cyst cells also with the transcript levels of *cabL1818*, *psaB* and *atpB* reduced to 0.68, 0.47 and 0.51-fold respectively of green vegetative cells. While transcript level of *lhcbm9* was being equal, *rbcL* was 3.46-fold more than that of non-stressed green cells (Figure 3.7).

3.3.2.3. Expression of photosynthetic genes and pigment production under stress conditions in the presence of photosynthetic inhibitors

The electron transport inhibitors DCMU and DBMIB were used to generate oxidizing and reducing states of the PQ pool. The cells were grown under HL for 2 days in the presence of these compounds. Since DBMIB is photosensitive, it is re-supplied at every 5-6 h. RT-PCR analyses showed the expression of *cabL1818* was increased to 1.73-fold when the PQ pool was oxidized by DCMU and reduced to 0.73-fold when PQ pool was reduced by DBMIB, whereas the expression of *lhcbm9* was reduced to 0.50-fold by DCMU and was equal to control when treated with DBMIB. Expression of *psaB* was up-regulated to 1.63-fold by DCMU and was equal to control by DBMIB treatment (Figure 3.8). The gene *rbcL* expression was reduced to 0.54-fold of control by DCMU while DBMIB addition partially recovered the reduction in expression to 0.74-fold of control. These results suggested that the expression of nuclear encoded photosynthetic genes, *cabL1818* and *lhcbm9*, and chloroplast encoded photosynthetic genes, *psaB* and *rbcL* are under the control of redox state of PQ pool.

The *atpB* gene expression was reduced to 0.51-fold by DCMU which was further reduced to 0.49-fold by DBMIB unlike other photosynthetic genes (Figure 3.8), indicating that the regulation of *atpB* gene expression may be mediated by other photosynthesis-derived signals. Both DCMU and DBMIB reduced the biomass production and Chl (a+b), while Chl a/b ratio was increased by DCMU and decreased by DBMIB. Total carotenoid, β-carotene and violaxanthin contents were increased

while lutein and neoxanthin contents were reduced by DCMU and DBMIB treatments (Table 3.4).

3.3.2.4. Influence of transcriptional, translational and carotenoid synthesis inhibitors on photosynthetic genes expression and pigment production

Actinomycin D reduced the expression of all photosynthetic genes studied (Figure 3.9), which substantiates that up-regulation of photosynthetic genes under stress conditions is due to over expression of mRNAs and not due to stability of mRNAs. Cycloheximide reduced the expression of photosynthetic genes (Figure 3.9), suggesting that their expression depends on de novo protein synthesis. Both total carotenoid and astaxanthin contents were reduced by actinomycin D and cycloheximide treatment (Table 3.4).

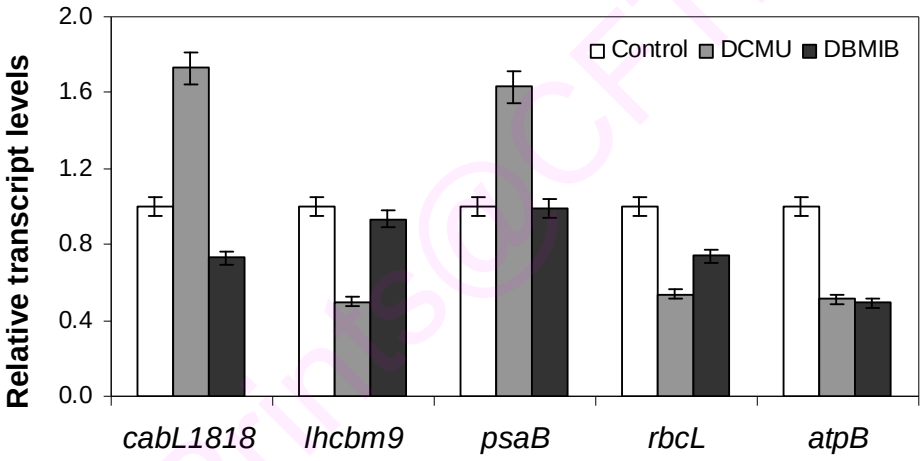


Figure 3.8. Influence of photosynthetic electron transport inhibitors, DCMU and DBMIB upon the expression of photosynthetic genes under stress conditions. Green motile cells were added with inhibitors and exposed to HL for 2 days. RNA extraction and RT-PCR were performed as described in the materials and methods with 1.5 µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean±SD of three independent experiments expressed as the fold increase in *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* genes of inhibitor added HL exposed cells compared with HL alone.

Chloramphenicol also reduced the expression of photosynthetic genes studied. The reduction in expression of *cabL1818* is less than other genes which is of 0.42-fold of control (Figure 3.9). The inhibitor treatment increased total carotenoid content, Chl (a+b), astaxanthin and lutein, and decreased β-carotene and Chl a/b ratio (Table 3.4).

Table 3.4. Pigment contents (mg g^{-1}) and biomass under the influence of inhibitors

Treatments	Astaxanthin	Lutein	β-carotene	Chl (a+b)	Chl a/b ratio	Total carotenoids	Biomass mg L^{-1}
Control	0.020 [a]	32.47 [a]	1.99 [ab]	31.07 [ab]	2.15 [a]	34.47 [a]	168.62 [a]
DCMU	0 [c]	28.62 [ab]	2.61 [a]	26.21 [b]	2.30 [a]	31.23 [a]	93.98 [c]
DBMIB	0 [c]	25.17 [ab]	2.13 [ab]	27.11 [b]	2.02 [ab]	27.31 [ab]	127.17 [b]
ActinomycinD	0.002 [c]	6.43 [cd]	0.23 [cd]	10.02 [d]	2.00 [ab]	6.68 [cd]	96.46 [c]
Cycloheximide	0.003 [bc]	7.13 [cd]	0.13 [cd]	19.16 [c]	1.03 [d]	7.27 [cd]	92.37 [cd]
Chloram-phenicol	0.008 [b]	33.44 [a]	1.29 [bc]	33.84 [a]	1.69 [bc]	34.81 [a]	153.12 [ab]
NF	0 [c]	18.74 [b]	1.48 [b]	24.12 [b]	1.98 [b]	20.22 [b]	158.36 [ab]

H. pluvialis cells are exposed to HL for 2 days without (control) or with the addition of inhibitors. Each value represents the mean of two separate experiments each with three replicates. Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

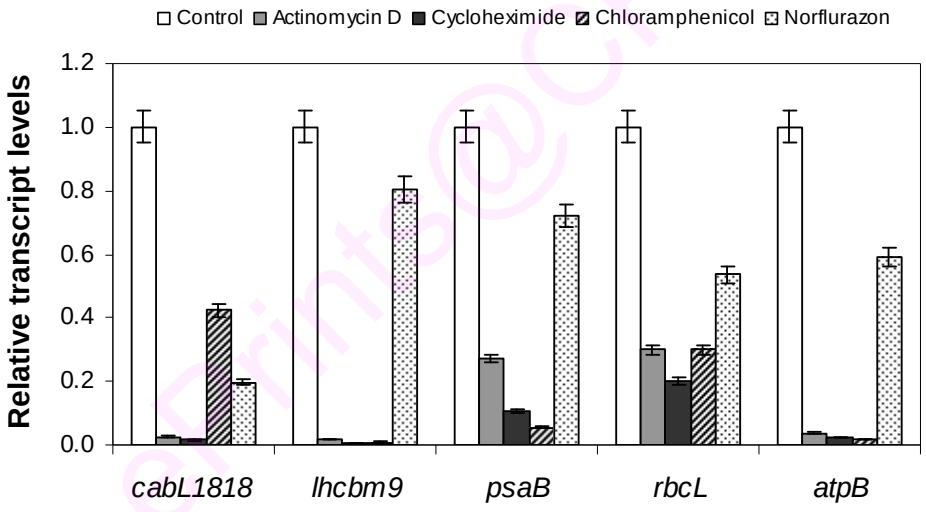


Figure 3.9. Influence of transcriptional, translational and carotenoids synthesis inhibitors upon the expression of photosynthetic genes under stress conditions. Green motile cells were added with inhibitors and exposed to HL for 2 days. RNA extraction and RT-PCR were performed as described in the materials and methods with 1.5 µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean ±SD of three independent experiments expressed as the fold increase in *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* genes of inhibitor added HL exposed cells compared with HL alone.

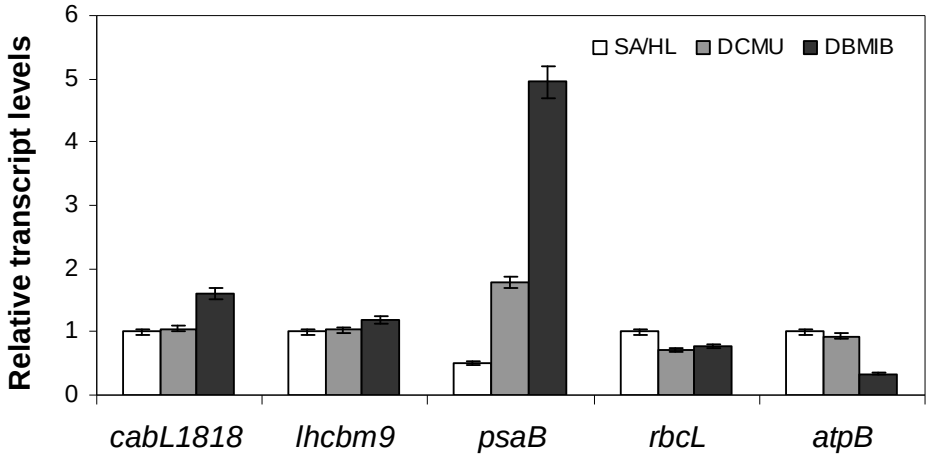


Figure 3.10. Influence of photosynthetic electron transport inhibitors, DCMU and DBMIB upon the expression of photosynthetic genes under stress induction by sodium acetate and high light (SA/HL). Green motile cells were added with inhibitors and exposed to SA/HL for 2 days. RNA extraction and RT-PCR were performed as described in the materials and methods with 1.5 µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean ±SD of three independent experiments expressed as the fold increase in *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* genes of inhibitor added SA/HL exposed cells compared with SA/HL.

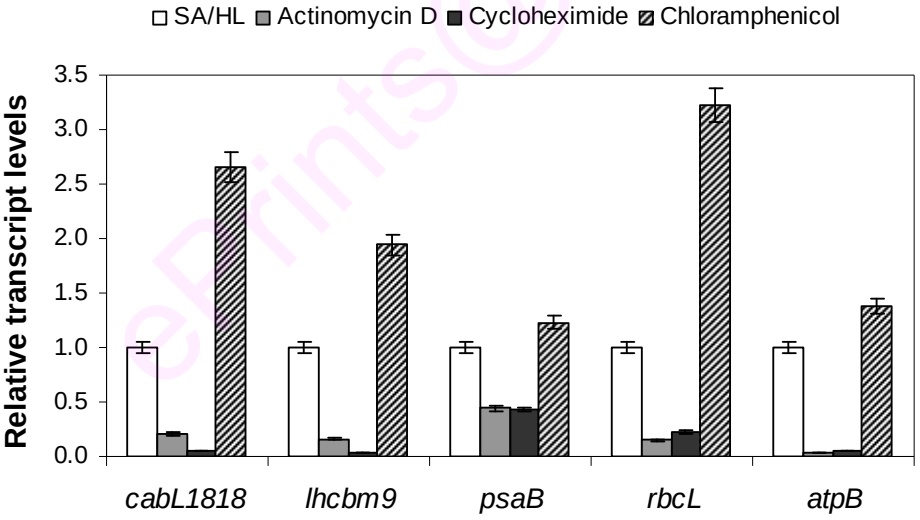


Figure 3.11. Influence of transcriptional and translational inhibitors on the expression of photosynthetic genes under stress induction by sodium acetate and high light (SA/HL). Green motile cells were added with inhibitors and exposed to SA/HL for 2 days. RNA extraction and RT-PCR were performed as described in the materials and methods with 1.5 µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The band intensity of each gene was adjusted with the band intensity of *Actin*. Data shown are mean ±SD of three independent experiments expressed as the fold increase in *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* genes of inhibitor added SA/HL exposed cells compared with SA/HL.

Carotenogenic inhibitor, NF is known to induce photo-oxidative stress in plants. Presence of this inhibitor reduced the expression of photosynthetic genes to 0.20, 0.80, 0.72, 0.54 and 0.59-fold of green cells for *cabL1818, lhcbm9, psaB, rbcL* and *atpB* respectively (Figure 3.9). NF inhibits phytoene desaturase so that the production of coloured carotenoids is affected. NF reduced Chl (a+b), Chl a/b, total carotenoid, lutein and β-carotene and inhibited astaxanthin production completely (Table 3.4). Treatments with transcriptional, translational and carotenoid synthesis inhibitors reduced biomass production (Table 3.4).

3.3.2.5. Acetate and high light induced changes in expression of photosynthetic genes

Addition of sodium acetate along with HL exposure (SA/HL) enhanced *lhcbm9*, *rbcL* and *atpB* expression to 3.38, 5.37 and 10.86-fold respectively of HL exposed cells. On the other hand *cabL1818* and *psaB* expressions were reduced to 0.74 and 0.18-fold of HL cells. Treatment with PET inhibitors in SA/HL cultures, DCMU and DBMIB increased the expression of *cabL1818* gene to 1.05 and 1.60-fold respectively of untreated control SA/HL, while *lhcbm9* gene expression was increased to 1.03 and 1.18-fold, and *psaB* expression was increased to 3.57 and 9.90-fold respectively. Both *rbcL* and *atpB* expressions were reduced by PET inhibitors. The reduction in expression of *rbcL* by DCMU and DBMIB was 0.70 and 0.77-fold respectively, while that of *atpB* was 0.93 and 0.34-fold respectively of SA/HL control (Figure 3.10). Expression studies in the presence of actinomycin D and cycloheximide in SA/HL exposed cells had shown reduction in expression of all photosynthetic genes studied. Plastid translation inhibitor, chloramphenicol enhanced the expression of photosynthetic genes (Figure 3.11).

3.4. Discussion

3.4.1. Regulation of carotenoid biosynthetic genes expression

The inhibition of astaxanthin and accumulation of β-carotene by DPA treatment observed in the present study is in accordance with the reports of Harker and Young (1995) and Grunewald and Hagen (2001). It has been found that the accumulated β-carotene is present in lipid vesicles (Grünewald and Hagen 2001). The reduction in β-carotene (0.57-fold of control) and lutein (0.51-fold) contents by nicotine suggests that lycopene β-cyclase which is a key enzyme in conversion of lycopene to β-carotene and ∝-carotene is affected by nicotine. The accumulation of lycopene by nicotine observed in the present study under NS/HL and ND/HL is in correlation with the report of Harker and Young (1995), who also observed the accumulation of lycopene under the influence of CPTA (2-(4-chlorophenylthio)-triethylamine HCl), a lycopene cyclase inhibitor. Similarly, the accumulation of lycopene by nicotine treatment was reported in other organisms. Eg. *Chlorella* (Ishikawa and Abe 2004) and *Xanthophyllomyces dendrorhous* (Ducrey Sanpietro and Kula 1998).

Cerulenin treatment inhibited astaxanthin synthesis and enhanced β-carotene accumulation in NS/HL condition (Figure 3.4). Similar to this, Schoefs et al. (2001) observed inhibition of astaxanthin without repressing de novo β-carotene synthesis under cerulenin treatment. Lipid biosynthesis inhibitor sethoxydim, which inhibits acetyl-CoA carboxylase, significantly decreased de novo fatty acid synthesis along with drastic inhibition of astaxanthin formation (Zhekisheva et al. 2005). Similarly, in *Dunaliella bardawil* which accumulates β-carotene as secondary carotenoid in lipid droplets of chloroplasts under stress condition, both lipid accumulation and β-carotene biosynthesis was inhibited under cerulenin and sethoxydim treatments (Rabbani et al. 1998). However under nutrient deficiency, astaxanthin accumulation increased in cerulenin treated cultures. This may be due to induction of TAG synthesis under nitrogen deficiency as reported by Zhekisheva et al. (2002).

Nicotine treatment reduced total carotenoid but it increased the accumulation of lycopene and enhanced the expression of all carotenoid genes studied. Similarly, up-regulation of the transcript levels of *PSY, PDS* and *LCY* were observed when daffodil flowers were treated with CPTA (Al-Babili et al. 1999). In contrast to our

study, they observed substantial increase in total carotenoids. Accumulation of phytoene was observed under NF treatment in *H. pluvialis* – both SC induced and non-induced (Harker and Young 1995) and in pepper (Simkin et al. 2000). Unlike the difference in expression of carotenogenic genes in the presence of NF observed in the present study, expression of *ZDS* (ζ-carotene desaturase), *PDS* and *PSY* genes were not affected by a decrease in colored carotenoids or by an accumulation of the precursors, phytoene or ζ-carotene in pepper leaves and seedlings due to a block in carotenoid biosynthesis (Simkin et al. 2000), and increase in total carotenoids appears to be independent of the regulation of the carotenoid genes transcript levels (Simkin et al. 2000). Inhibition of carotenoid synthesis in tomato seedlings by bleaching herbicides was accompanied by an increase in *PSY* and *PDS* mRNA levels (Giuliano et al. 1993). Decreased transcript levels of the *PSY* gene with phytoene accumulation was observed in sunflower (Campisi et al. 2006) and in *Arabidopsis thaliana*, *PDS* expression was found to be independent of leaf pigment content (Wetzel and Rodermel 1998).

Expression of carotenogenic genes under ND condition compared to NS condition under high light revealed that expression of *PSY*, *PDS*, *LCY*, *BKT* and *CHY* were 17.84, 1.09, 3.49, 2.73 and 2.84-fold higher respectively (Figure 3.5). Earlier studies on analysis of carotenoid gene transcripts in *H. pluvialis* indicated the up-regulation of *PSY* and *CHY* transcripts by HL, sodium acetate and NaCl (Steinbrenner and Linden 2001), *PSY*, *PDS*, *LCY* and *CHY* under HL (Steinbrenner and Linden 2003) in heterotrophic grown cultures, *PDS* and *BKT* by nitrate deprivation (Grünewald et al. 2000), *CHY* in cyst cells (Linden 1999) and *BKT* genes by HL, sodium acetate and, sodium acetate and Fe^{2+} (Huang et al. 2006a). All the inhibitors showed enhanced expression of carotenogenic genes under ND compared to respective inhibitor condition under NS except *LCY* and *BKT* of nicotine and *PSY* and *LCY* of cerulenin treated cells (Figure 3.6). The decrease in *BKT* expression by nicotine was not significant. Reduced expression of *LCY* in nicotine treated cultures was reflected in increased lycopene accumulation in ND compared to NS. Moreover the decreased *PSY* and *LCY* in cerulenin treatment correlated well with decreased β-carotene and lutein, and corresponding increase in astaxanthin by increased *BKT* and *CHY* expression (Figure 3.6).

In the present study all the inhibitors studied resulted in decreased carotenoid content in *H. pluvialis* cells under secondary carotenoid inducing conditions. There was no correlation observed between the enhanced transcript levels of carotenogenic genes and decreased total carotenoids which may be explained as follows. The decreased translation might have increased the stability of these genes as observed for chloroplast genes in the presence of chloramphenicol (Kato et al. 2006). Among the inhibitors studied nicotine enhanced all the carotenogenic genes expression while DPA and NF decreased the expression of *PDS*, *BKT* and *CHY*. *PDS* was inhibited by cerulenin while *PSY* was inhibited only by DPA. However, all the inhibitors decreased the total carotenoid content. It is also interesting to note that total carotenoid content was significantly less in nicotine treated cultures (0.53%) in which all the carotenogenic genes exhibited enhanced levels of expression (Table 3.3). This clearly states that these inhibitors are likely to act at post translational or enzyme level in addition to their action at transcriptional level in the carotenoid biosynthesis pathway. Lycopene accumulation in the nicotine treated cultures further substantiates the above statement. The increase in total carotenoids was reported in higher plants in the presence of inhibitors due to accumulation of precursors (Al-Babili et al. 1999). However in the present study the decrease in total carotenoids observed in spite of the accumulation of precursors, may be due to the higher rate of degradation of carotenoids in *H. pluvialis* upon exposure to high light in the absence of astaxanthin which is likely to have photo-protective role. The detailed study of carotenoid gene transcripts and carotenoid content described in this article indicates that, in addition to the transcriptional regulation of carotenoid biosynthesis, regulation at other levels appear to be operational.

3.4.2. Regulation of photosynthetic genes expression

Photosynthesis plays an important regulatory role in the acclimation of photosynthetic organisms to light conditions (Allen and Pfannschmidt 2000; Steinbrenner and Linden 2003). This acclimation involves changes in the redox state of the components of the photosynthetic electron transport that leads to the adjustment of photosystem stoichiometry and to balance electron transport rates. Redox control was also reported to be involved in the post-translational modification of the photosynthetic proteins, and regulation of transcription of chloroplast and nuclear genes for photosynthesis (Escoubas et al. 1995; Pfannschmidt et al. 1999), post-transcriptional regulation of

chloroplast genes (Alexciev and Tullberg 1997) and other genes (Kujat and Owttrim 2000). Our previous study has shown, *H. pluvialis* carotenoid synthesis genes are up-regulated during stress induction by high light and nutrient deficiency and are regulated at transcriptional, post-transcriptional and translational levels (Vidhyavathi et al. 2008). Light induction of *H. pluvialis* carotenoid genes is also reported to be under photosynthetic redox control (Steinbrenner and Linden 2003).

In *H. pluvialis*, exposure to high light intensities induces decreased Chl (a+b) and increased Chl a/b ratio (Table 3.3). The increased Chl a/b ratio upon exposure to high light and high light/nitrogen deficiency was reported by Kitajima and Hogan (2003), and reduced Chl a/b ratio was observed by Zlotnik et al. (1993) in *H. pluvialis* during aplanospore formation in nitrogen deficient medium. In addition, the loss of Chls and reduction in Fv/Fm ratio (variable to maximum fluorescence, maximum photochemical yield of PSII) observed in the present study under stress conditions (Table 3.3) indicated the stress response leads to photo inhibition (Torzillo et al. 2005), but further reduction in Fv/Fm of cysts (Table 3.3) indicated that cyst cells are photosynthetically competent but they operate at photosynthetically reduced level (Vidhyavathi et al. 2007), which may be due to impaired linear electron flow from PSII to PSI (Tan et al. 1995). In *H. pluvialis*, quenching of excess light under secondary carotenoid inducing conditions are done by xanthophyll-dependent quenching for first few hours of exposure to high light and later light is filtered by bright red color of astaxanthin (Masojidek et al. 2004). This photo-protective nature of astaxanthin might be protecting the photosynthetic apparatus from excess light and maintaining reduced photosynthetic activity in cysts.

Figure 3.7 shows photosynthetic genes, *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* were up-regulated by HL and ND/HL induced stress with each gene following different kinetics of up-regulation. This indicated that expressions of these genes are transcriptionally regulated, and transcripts of these genes were found even in cyst cells. Li and Sherman (2000) also reported enhanced accumulation of *psbA* and *psbDII* transcripts in *Synechocystis* sp. strain PCC 6803, by a shift to high light conditions, to compensate for the loss of D1 and D2 proteins and to maintain a functional PSII. The up-regulation of photosynthetic genes observed in the present study, were correlated well with the observations of Wang et al. (2004a) and Kim et al. (2006) in the proteomics studies of *H. pluvialis*. Wang et al. (2004a) reported that Rubisco large subunit, ATP synthase α-subunit and ATP β-subunit proteins are

transiently down-regulated, then up-regulated under oxidative stress induced by 45 µM Sodium acetate, 450 µM ferrous sulfate and continuous illumination of 150 µmol photons m^{-2} s^{-1}. Kim et al. (2006) observed that light harvesting Chl *a/b* binding protein associated with PS I, and ATP synthase α-subunit proteins are induced in red cyst cells of *H. pluvialis* under high light stress. In contrast to our results, most of the photosynthetic genes of *H. pluvialis* are down-regulated under nitrogen deficiency and high light stress (Eom et al. 2006). Expression of *Lhcb* genes in *Chlamydomonas* (Teramoto et al. 2002) and *LhcII* genes of *Dunaliella salina* (Wei et al. 2007) are down-regulated by high light. In *Chlamydomonas reinhardtii*, the abundance of *Lhcb*, *Lhcb4* and *Lhca* transcripts were reduced within 1-2 h of high light exposure, and they recovered to their low light concentrations within 6-8 h (Dunford et al. 2003).

Figure 3.8 shows the opposite effects of DCMU and DBMIB on the expression of nuclear encoded photosynthetic genes, *cabL1818* and *lhcbm9*, and chloroplast encoded photosynthetic genes, *psaB* and *rbcL*, suggesting that these genes are under the control of redox state of PQ pool. In many organisms expression of photosynthetic genes are under photosynthetic redox control. For example, in *Dunaliella* spp., the down-regulation of *cab* by high light is under redox control (Escoubas et al. 1995; Maxwell et al. 1995), like wise the transcription of *cab* and *rbcS* genes of *Arabidopsis* (Oswald et al. 2001), *psaB* gene of pea (Tullberg et al. 2000), *psaA*/*B* and *psbA* of mustard (Pfannschmidt et al. 1999) are also under redox control. Inhibition of high light induced expression of *atpB* mRNAs by both DCMU and DBMIB (Figure 3.8), shows that the redox state of the PQ pool is not involved in this situation. As suggested by Alfonso et al. (2000), cytochrome *b6/f* complex may be involved in the regulation of *atpB* gene expression. In *Synechocystis* sp. 6803, the redox state of the cytochrome *b6/f* complex appeared to regulate the transcription of a PSI gene, *psaE* (El Bissati and Kirilovsky 2001).

Studies using actinomycin D, a transcriptional inhibitor, had shown reduction in expression of photosynthetic genes, *cabL1818, lhcbm9, psaB, rbcL* and *atpB* upon exposure to inhibitor (Figure 3.9). This substantiates that the high light induced up-regulation of these genes is due to active transcription and not due to stability of their mRNAs. This is similar to earlier reports on low stability of photosynthetic genes (Brinker et al. 2001). Likewise, transcripts of these genes were reduced in the presence of cycloheximide, a cytoplasmic translational inhibitor (Figure 3.9), suggesting these genes expressions were dependent on de novo protein synthesis in

the cytosol. This is in concordance with the report of Teramoto et al. (2004), that HL-induced expression of *Lhl4* required de novo protein synthesis in the cytosol. The reduction in expression of photosynthetic genes under the influence of transcription and cytoplasmic translation inhibitors (Figure 3.9) was correlated well with the reduction in photosynthetic pigments (Table 3.4). In contrast to our observation of decreased expression of photosynthetic genes in the presence of chloramphenicol, increased accumulation of *psaB* and *psaA*, and normal accumulation of *rbcL* and *atpB* transcripts were observed in *Chlamydomonas* (Xu et al. 1993). Similar to our results, the increased accumulation of Chl and lutein, and decreased accumulation of *lhcp* mRNAs in chloramphenicol treated wheat chlorina mutant (Mogen et al. 1990) and loss of β-carotene and increased xanthophyll cycle pool pigments in *C. reinhardtii* (Depka et al. 1998) were observed.

Blocking of carotenoid biosynthesis either through mutation or by growth in the presence of bleaching herbicide NF, a noncompetitive inhibitor of carotenoid biosynthesis, would be useful in generating carotenoid deficient condition for studying biochemical and molecular changes. Under these conditions, high light intensities cause bleaching and photo-oxidation (Sagar et al. 1988). Growth of *H. pluvialis* in the presence of NF under high light showed decreased expression of photosynthetic genes, affecting chloroplast genes more than nuclear genes (Figure 3.11). Similar to our results, reduction in expression of *atpB* and *psaA* genes were observed in NF treated young spinach leaves (Tonkyn et al. 1992) and complete absence of *Lhcb1* mRNA in NF-treated maize leaves (La Rocca et al. 2000). Like DCMU and chloramphenicol or lincomycin, NF also has primary effects on plastid-located processes (Polle et al. 2000). The decreased expression of photosynthetic genes in the presence of NF observed in the present study (Figure 3.9) indicated the role of some other plastid signal in the regulation of photosynthetic genes expression. It was reported that Mg-protoporphyrin and its monomethylester may act as plastid signal for reducing photosynthetic genes expression under NF treated condition (La Rocca et al. 2001).

Acetate enhances astaxanthin production in *H. pluvialis* through transcriptional up-regulation and post-transcriptional modification of carotenogenic genes (Vidhyavathi et al. 2008). To find out the effect of acetate on the expression of photosynthetic genes, cells were exposed to HL for 48 h after the addition of acetate with or without the addition of PET inhibitors. Acetate modulates HL induced

expression of photosynthetic genes. Similar to our results, decreased *psbA* and slightly increased *psaA* transcripts were observed in *Chlamydobotrys stellata* in the presence of acetate (Kovacs et al. 2000). In contrast to our observation on up-regulation of *lhcbm9* by acetate, the down regulation of *lhcb* was observed by Kindle (1987). Acetate induced up-regulation of *lhcbm9* was further enhanced by PET inhibitors, while the reduction in *psaB* expression was recovered by inhibitors addition. For both *rbcL* and *atpB*, acetate induced enhancement in transcript levels were reduced by PET inhibitors (Figure 3.10). Since DCMU and DBMIB have opposite effects on the redox state of the PQ pool, it can be concluded from the present study that redox state of PQ pool is not involved in acetate induced differential expression of photosynthetic genes, and they may be regulated at redox state of cytochrome *b6/f* complex. It was reported that, acetate is involved in influencing photosynthetic activity by reducing PQ (Endo and Asada 1996) and is involved in regulation of the redox state of photosystem components thereby affecting transcription of nuclear and chloroplast photosynthetic genes (Kovacs et al. 2000). Results from the present study suggested acetate is involved in the regulation of photosynthetic genes transcription mainly through the redox state of the cytochrome *b6/f* complex. Both actinomycin D and cycloheximide, reduced SA/HL induced expression of photosynthetic genes, suggests acetate induced expression is not due to increased mRNA stability and it depends on de novo protein synthesis. Though the reason is not clear, chloramphenicol treatment in SA/HL exposed cells not only increased the expression of chloroplast genes but also nuclear genes of photosynthesis (Figure 3.11). The reason may be increased stabilization of photosynthetic gene transcripts by reduced translation (Kato et al. 2006) in the presence of acetate. Our previous study also suggested involvement of acetate in the post-transcriptional modification of carotenoid genes in *H. pluvialis* (Vidhyavathi et al. 2008).

Present study is a first report of its kind in *H. pluvialis*, where photosynthetic genes were studied under stress conditions which induce secondary carotenoid synthesis and their regulation was also studied at PET, transcriptional and translational levels and under photo-oxidative stress condition. The results showed photosynthetic genes were regulated at transcriptional level and high light induced differential expression of photosynthetic genes were regulated by redox state of PQ pool but for *atpB* which was regulated by cytochrome *b6/f* complex. Enhanced expression observed for few genes are due to enhanced transcription not due to

stability, and expression of these photosynthetic genes depends on cytoplasmic and plastid protein synthesis. Acetate modulates the HL induced expression of photosynthetic genes and this depends on redox state of cytochrome *b6/f* complex and cytoplasmic protein synthesis. Blocking of plastid translation enhanced stability of photosynthetic genes in acetate treated cultures. From the present study, it may be inferred the involvement of multiple signals in modulating the expression of photosynthetic genes under secondary carotenoid inducing conditions. Moreover, from our previous study (Vidhyavathi et al. 2008) and present results, it can be suggested that both photosynthetic genes and carotenoid genes are coordinately regulated in *H. pluvialis* under stress conditions. In addition, *H. pluvialis* would be a model organism to study the mechanism of light-harvesting and photo-protection under secondary carotenoid inducing conditions.

Chapter IV

Molecular and biochemical changes associated with carotenogenesis

Summary

The genes differentially expressed under the stress conditions were analyzed using the mRNA differential display-RT PCR (DD-RT PCR) technique. From two populations of mRNA (green vegetative cells and cells exposed to high light intensity 97 µmol m^{-2} s^{-1}), a total of 109 transcripts which were differentially expressed under stress conditions have been identified. After removal of false positives by re-amplification, 34 partial cDNAs were cloned, sequenced and analyzed *in silico*. Almost 50% of the DD cDNAs were either of unknown function or shared no apparent homology to any expressed sequences in the GenBank/EMBL databases. And about 50% of DD clones were similar to molecules related to general metabolism, photosynthesis, carotenoid synthesis, lipid synthesis, tetrapyrrole synthesis, transporter proteins, defense signaling and genetic information processing. The differential expression patterns were confirmed by RNA dot blot, northern blot and semi-quantitative RT-PCR analyses. RNA analyses of 6 DD clones revealed that 4 genes were induced under high light stress and two genes exhibited reduced expression. Changes in pigment profile with reference to carotenoid composition in different cell fractions, activities of carotenoid enzymes such as LCY and BKT, antioxidant enzymes, protein content and influence of phytohormones salicylic acid (ScA) and methyl jasmonate (MJ) were studied. LCY and BKT activities were found to increase under stress condition. Maximum activity of BKT was observed in lipid globules of stress induced cultures. Analysis of astaxanthin composition revealed decrease in free astaxanthin and increase in astaxanthin mono and diesters. Influence of CO_2 enrichment (2%) along with other stress conditions like high light, nutrient deficiency, NaCl and sodium acetate addition on carotenoid accumulation was studied. Under the influence of high light, exposure of cells to nutrient deficiency enhanced carotenoid accumulation which was further enhanced by exposure to CO_2 enriched environment and/or NaCl and sodium acetate addition. Changing the carbonate buffer in the lower compartment of 2-tier vessel to provide constant CO_2 environment was found to be efficient in enhancing carotenoid content. A maximum of 30.61 mg g^{-1} of total carotenoid with 87% astaxanthin was achieved by exposing cells to high light, NaCl and CO_2. ScA and MJ are exerting varied response in antioxidant systems of *H. pluvialis*, possibly due to different mechanisms of induction of antioxidant components against signaling molecule induced oxidative stress. Higher concentrations of ScA and MJ inhibited astaxanthin accumulation but each is having different mechanism of inhibition either by scavenging the free radicals or by increasing primary carotenoids production.

4.1. Introduction

Astaxanthin functions as an antioxidant agent against oxidative stress (Kobayashi 2000; Li et al. 2008b) and offers protection against photo-oxidative damage and photoinhibition (Wang et al. 2003b). Recent genomic and proteomic studies in *H. pluvialis* to investigate global gene and protein expression revealed that many genes and proteins related to growth, photosynthesis and respiration are differentially expressed in addition to up-regulation of carotenoid genes and enzymes, during secondary carotenogenesis (Hu et al. 2003; Wang et al. 2004a, 2004b; Eom et al. 2005). Such global analysis of carotenogenesis process would be helpful for identification of differentially expressed novel genes and gene products since it is possible that some of the gene products may be involved in regulation of the stress response mechanisms in *H. pluvialis*.

Transcriptome profiling of plants to environmental stresses can be studied using different techniques, which include differential display reverse transcription PCR (DDRT-PCR), serial analysis of gene expression (SAGE), subtractive hybridization, DNA-chip, and cDNA microarray. mRNA differential display is a powerful tool for identifying and cloning genes whose expression levels have been altered under different environmental conditions because of its technical simplicity and lack of requirement for previous genomic information of the species of interest (Liang and Pardee 1992; Carginale et al. 2004). Differential display involves the random amplification of cDNA transcripts to identify particular mRNA species that are expressed under defined conditions, and it enables very small differences in transcription patterns to be detected. DD has been used by many people to find differentially expressed transctripts under stress conditions (Im and Grossman 2001; Vranová et al. 2002). Even in *H. pluvialis* using DD technique, a transcript which is homologue to prokaryotic htrA was identified which is reported to be differentially expressed during different development stages. This mRNA is not detectable in vegetative cells, but was found at high levels in developing aplanospores (Hershkovits et al. 1997).

Under normal condition, reactive oxygen species (ROS) are inevitably generated through the electron transport activities in chloroplasts, mitochondria and plasma membrane. Detoxification of ROS involves antioxidant defense mechanisms

which include several enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), carotenoids, flavonoids, phycocyanin and ascorbate etc (Mallick 2004). Phytohormones such as abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid (ScA) appear to be critical components of complex signaling networks in defense responses (Tarakhovskaya et al. 2007; Walia et al. 2007) and have diverse functions in plant systems. Essentially all known phytohormones of higher plants were found in algae but the information about the hormone metabolism and action mechanisms is scarce (Tarakhovskaya et al. 2007). Involvement of both ScA and methyl jasmonate (MJ) on growth and biochemical activity of *Chlorella vulgaris* was observed (Czerpak et al. 2002; Czerpak et al. 2006). In *Scenedesmus incrassatulus*, MJ is involved in changing the response of organism to salt stress (Fedina and Benderliev 2000). Effects of methyl jasmonate (MJ) have also been seen on *Euglena gracilis* Klebs and in red and brown macroalgae (Ueda et al. 1991), suggesting a widespread use of this signaling molecule in photosynthetic organisms. In *Fucus* (Phaeophyta), JA and MJ are reported to be important components of the signaling pathway resulting in the development of defensive responses after plant damage with phytophages (Arnold et al. 2001). In plant cell cultures, ScA and MJ are reported to enhance secondary metabolite production (Sudha and Ravishankar 2003) through influence of transcription of some nuclear genes (Stockigt et al. 1995).

Hence the present study has been taken to study the molecular and biochemical changes associated with carotenogenesis in *H. pluvialis*. Differentially expressed cDNA transcripts under stress conditions were identified by using DDRT-PCR technique. Expression analyses of selected transcripts were carried out. In addition to biochemical changes, effect of CO_2 enrichment and phytohormones were studied.

4.2. Materials and Methods

4.2.1. Differential display of light regulated transcripts in *H. pluvialis*

4.2.1.1. Culture growth

H. pluvialis cells grown for 7 days (green motile cells) and cells exposed to high light intensity (HL) of 97 μmol m^{-2} s^{-1}, nutrient deficiency and high light (ND/HL), nutrient deficiency, high light and 0.1% NaCl (ND/HL/NC), nutrient deficiency, high light and 4.4 mM sodium acetate (ND/HL/SA), and nutrient deficiency, high light, NaCl and sodium acetate (ND/HL/NC/SA) were used for analysis.

4.2.1.2. Growth and pigment analyses

Algal cell number was determined by counting algal cells using Neubauer haemacytometer. Known volume of culture was centrifuged at 3000 × *g* for 10 min and dry weight of the biomass was estimated as described in section 2.2.5.2. An aliquot of culture was harvested and freeze dried. Pigment extraction and analyses were carried out as per the procedure given in section 2.2.6.

4.2.1.3. RNA isolation and removal of genomic DNA contamination

Total RNA extraction and possible genomic DNA contamination removal was carried out as described in section 2.2.7.

4.2.1.4. mRNA differential display

The mRNA Differential Display was conducted in two RNA samples namely RNA isolated from green vegetative cells and cells exposed to HL stress. For cDNA synthesis 0.2 μg of total RNA was used. Briefly, RNA (0.2 μg) was heated at 70°C for 5 min and immediately chilled on ice. First-strand cDNA synthesis was performed in a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1mM each dNTP, 40 units of RiboLock™ Ribonuclease Inhibitor (MBI Fermentas GmbH, St. Leon-Rot) and 40 units of H-minus M-MuLV Reverse Transcriptase (MBI Fermentas) and 50 mM of different anchor primer (Table 4.1) for 1 h at 42°C. The reaction was stopped by heating the mixture at 70°C for 10 min and immediately chilled on ice. Later, second strand cDNA amplifications were performed using total of 5 arbitrary primers in all 35 combinations with 7 different anchored primers. PCR mixture (25 μl) contained 2 μl of reverse transcribed cDNA,

1X PCR buffer (Fermentas GmbH, St. Leon-Rot), 200 µM dNTPs (Fermentas GmbH, St. Leon-Rot), 1 units of Taq DNA polymerase (Bangalore Genei, Bangalore), 1 µM of each arbitrary primer (GenHunter, TN) and 1µM of respective anchored oligo (dT)-primer (Table 4.1). The PCR was performed at an initial denaturation at 94°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and 2 min extension at 72°C with a final extension at 72°C for 10 min using a thermal cycler (MWG Peqlab, Germany).

Table 4.1. Different anchored Oligo dT primers and arbitrary primers used for differential display

Anchored primers 5' – 3'	Arbitrary primers 5' – 3'
DD1 - TTTTTTTTTTTAA	OPA20 - GTTGCGATCC
DD2 - TTTTTTTTTTTAC	OPD07 - TTGGCACGGG
DD3 - TTTTTTTTTTTAG	OPF15 - CCAGTACTCC
DD4 - TTTTTTTTTTTCA	OPJ12 - GTCCCGTGGT
DD5 - TTTTTTTTTTTCC	AP3 - AAGCTTTGGTCAG
DD6 - AAGCTTTTTTTTTTTA	
DD7 - AAGCTTTTTTTTTTC	

After selective amplifications, a 6 µl aliquot of PCR amplified products was mixed with equal volume of denaturing loading buffer (98% formamide, 10 mM EDTA; 0.05% Xylene–cyanol, 0.05% Bromo-phenol blue), denatured at 90°C for 3 min and immediately cooled on ice. Samples (12 µl) were loaded onto pre-warmed denaturing 6% polyacrylamide standard sequencing gel of 28:2 ratio acrylamide:bisacrylamide, 7.5 M urea, 1 X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0), then electrophoresed in 1 X TBE buffer at 50 W constant power for about 2 h and 30 min; until the loading dye reached the bottom of the gel. The gel was fixed by incubating the gel slab in fixation solution (2% ethanol and 0.1% acetic acid) for 10 min with gentle shaking. Then the gel was rinsed with distilled water twice for 5 min each and incubated in staining solution (chilled 0.2% $AgNO_3$ prepared in fixation solution) for 20 min followed by brief wash in double distilled water for 10 sec. The gel was developed with developing solution (0.6% NaOH and 0.2% of 37% formaldehyde). The developing solution was discarded as soon as it turns yellow and replaced it with a fresh portion. When a sufficient degree

of staining has been obtained, developing solution was replaced with 5% acetic acid and then the gel was washed with distilled water.

4.2.1.5. Sequencing and homology search

Differentially expressed bands were excised from the gel and extracted using QIAEX II polyacrylamide gel extraction kit (Qiagen) according to manufacturer's instructions. For reamplification, 2 µl of this solution was used in standard PCR reactions using the same primer pair used in the Differential Display of the correspondent reaction. PCR reactions were prepared in a final volume of 20 µl containing 1 X Taq buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 9.0), 0.25 mM dNTPs, 0.5 µM each primer and 1 unit of Taq DNA polymerase enzyme (Biotools, Spain). The cycle program consisted of an initial step of 4 min at 94°C, 35 cycles of 30 s at 94°C, 60 s at 40°C and 45 s at 72°C, followed by a final extension step of 8 min at 72°C. Directly after PCR amplification, the reaction mixture was heated at 95°C for 20 min. Then 1 µl of 2 mM dATP/ 10 µl of PCR product was added and incubated at 70°C for 15 min. Then the PCR products were purified using HiPura PCR clean-up kit (HiMedia, India). A-addition was performed using A-addition kit from Qiagen according to the manufacturer's instructions and cloned into pKRX-T vector following the cloning methodology (SBS Biotech, Beijing, China) according to the supplied protocol. Ligated products were transformed into competent cells of *Escherichia coli* strain DH5α. Plasmids from recombinant clones were isolated with the Qiagen mini prep Plasmid Mini Kit and sequenced using universal primers with commercial services (MWG Biotech, Bangalore, India and Bioserve, Hyderabad, India). Homology search was carried out using the NCBI BLASTX and BLASTN program with amino acid and nonredundant nucleotide sequences respectively.

4.2.1.6. RNA dot blot

The total RNA was precipitated overnight at 4°C by the addition of 0.3 volumes of 10 mM LiCl and then precipitated in ethanol. The quality and quantity of the RNA was checked by electrophoresis as described in the earlier section. One µl of total RNA (RNA from the stock 1 µg μl^{-1}) was denatured with 2 volumes of denaturing buffer (1X MOPS containing formamide and formaldehyde) by boiling for 5 min and immediately chilled on ice and spotted to a Hybond-N nylon membranes (Ambion).

The RNA was fixed to the membrane by using 302 nm ultraviolet cross linker (Ambion) for 45 min. Then the membranes were pre-hybridized for 1 h in ULTRAHyb buffer (Ambion) and hybridization was then performed overnight with the same buffer containing the gene-specific DIG-labeled probe at 42°C. The probe was prepared by the amplifying 318 bp cDNA fragment using primer specific to H97.1 (Table 4.2). Then amplified products were purified and labeled by DIG High prime method as described by manufacturer's instruction (Roche). After hybridization, the blots were washed with 2.0 × SSC / 0.1% SDS for 15 min at RT and finally with 0.1 × SSC / 0.1% SDS for 15–30 min at 65°C. The blots were washed and visualized by following manufacturer's instructions (Roche).

Table 4.2. Oligonucleotide primer sequences and PCR conditions used for the confirmation of differentially displayed products with quantitative RT-PCR

DD product	Primer sequence (5'–3')	Amplicon size (bp)	Annealing temperature °C	Cycle numbers
H7.1 F	TTGCAACAAGAGACGGAAGA	214	56	27
H7.1 R	CGCCGGAGAACAAGGTACT			
H8.1 F	TGCAGTAGATGTAAGGCAACG	286	56	27
H8.1 R	CGACACAGCAACACAGGAGA			
H13.2 F	CACGGGTAGGTTCATCAAGG	408	60	28
H13.2 R	CAGGTCGTACCGAACTGATG			
H53.1 F	GCTTAAGGCTGGTGAATTCCT	300	56	27
H53.1 R	ATTAATGCAGCTGGCACGAC			
H69.1 F	TGGCAAGGGTTGTAGTGACA	179	56	27
H69.1 R	CGTGGTGTATTTGGACTGGA			
H97.1 F	CAAGGACGCATACCTAAACG	318	60	28
H97.1 R	TGCGATCCCAACTTTATTAACTG			
ACTIN F	AGCGGGAGATAGTGCGGGACA	200	61	28
ACTIN R	ATGCCCACCGCCTCCATGC			

4.2.1.7. Northern blot

RNA blots were prepared according to standard protocols (Sambrook et al. 1989) using 0.1 μg of total RNA per lane. Total RNA from green vegetative and high light exposed cells were resolved on 1.2% agarose gels containing 7% formaldehyde, transferred onto nylon membrane (Ambion) and hybridized with digoxigenin-labeled DNA probes in DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany). DIG labeled probes were generated by DIG-HIGH Prime labeling and

detection starter kit I. After hybridization, the blot were washed with 2.0 × SSC / 0.1% SDS for 15 min at RT and finally with 0.1 × SSC / 0.1% SDS for 15–30 min at 65°C. Then the blots were washed and visualized by following manufacturer's instructions.

4.2.1.8. Reverse transcription-polymerase chain reaction (RT-PCR)

First strand cDNA synthesis was performed in a 20-µl reaction volume using 1.5 µg of total RNA, oligo dT_{18} and M-MLV reverse transcriptase (Ambion) according to manufacturer's protocol. The reaction was allowed to proceed for 60 min at 42°C before being terminated by treatment at 92°C for 10 min. The primers used for amplification and amplification conditions are listed in Table 4.2. RT-PCR was carried out as described in section 2.2.7. Following the separation of the PCR products on ethidium bromide-stained 1.4% agarose gels, the bands were quantified. Each band was normalized against the intensity obtained with the same cDNA using the *ACTIN*-specific primers. For calculating the transcript abundance under stress conditions, the transcript levels of each gene in green motile cells were taken for comparison.

4.2.2. Biochemical changes associated with carotenogenesis

4.2.2.1. Carotenoid enzyme activities

4.2.2.1.1. Culture growth

H. pluvialis green motile cells and cells exposed to high light (HL) for 2 days were harvested for enzyme assays.

4.2.2.1.2. Determination of β-carotene ketolase activity

Preparation of Cell Fractions. Aliquots of cells were harvested by centrifugation at 1,400 × g for 2 min and re-suspended in break buffer (0.1 M Tris-HCl pH 6.8, 5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 5 mM Na_2-EDTA, 0.3 M sorbitol, and 0.1 mM phenylmethylsulfonyl fluoride). The suspension was sonicated for 20 s in a homogenizer (Sonopuls Ultrasonic Homogenizer HD 2200, Bandelin electronic GmbH and Co. KG) and then centrifuged for 10 min at 16,000 × g to yield chloroplast and cell debris pellet. The supernatant was centrifuged at 16,000 × g for 10 min to yield chloroplast fraction. The supernatant was transferred to a fresh tube and centrifuged at 90,000 × g (Beckman L7-65 ultracentrifuge, USA) for 1 h and the lipid

vesicles floating on top were separated. The chloroplast and lipid fractions were stored at -20°C and used for β-carotene ketolase activity and pigment estimation.

Enzyme assay. β-carotene ketolase activity was determined by the method of Fraser et al. (1998). Lipid suspension was prepared by resuspending 10 mg of soybean oil in 1 ml of chloroform and centrifuged at 12000 × g for 5 min. The clarified solution was placed in a clear round-bottomed test tube and the lipid was dried onto the surface of the glass tube under nitrogen. 100 mM Tris-HCL pH 8.0, containing 1 mM dithiothreitol (0.5 ml) was placed onto the residue. Sonication (3×2-s bursts, full power) was done to yield a suitable lipid suspension. Lipid vesicles were prepared freshly prior to incubations. The final volume of the reaction mixture used was typically 600 µl. The cofactor solution (295 µl) contained dithiothreitol (1 mM), $FeSO_4$ (0.5 mM), ascorbic acid (5 mM), 2-oxoglutarate (0.5 mM), and deoxycholate (0.1% mass/vol.). Lipid suspension (20 µl) and cell extract (300 µl) were added to the cofactor solution, mixed and then equilibrated at 30°C for 5 min. Finally the reaction was initiated by the addition of 6 µl of β-carotene in chloroform (1.0% by vol.). Control incubations with buffer (0.4 M Tris-HCl, pH 8.0) alone and boiled (3 min) cell extracts were also performed. The mixture was incubated by shaking in the dark for 4 h at 30°C. Incubations were terminated by the addition of methanol and stored at -70°C.

Products and substrates from the *in vitro* incubations were extracted with 3 volumes of 10% (v/v) diethyl ether in petroleum ether 40-60°C. After mixing a partition was formed by centrifugation at 3000 × g for 5 min at 4°C. The organic phase was removed and the aqueous phase re-extracted with the same solvent. The remaining aqueous phase was further re-extracted with chloroform (2 volumes). The organic extracts were pooled and brought to dryness under a stream of nitrogen, and stored in -70°C. HPLC analysis was performed as described in section 2.2.6.4.

4.2.2.1.3. Determination of lycopene cyclase activity

Lycopene cyclase activity in the *H. pluvialis* cells was determined by the method of Schnurr et al. (1996). The culture was centrifuged and the wet biomass was extracted with 0.2 M Tris-Maleate buffer (pH 6.8). The extract was centrifuged and supernatant was used for enzyme assay. The reaction mixture (final volume 0.5 ml) contained 50 µl of 5 mM NADH and 150 µl of lycopene in soybean oil (as substrate) and 300 µl of

enzyme. The incubation was done in dark at 30°C for 4 h. Reaction was terminated by adding methanolic KOH. The mixture was extracted with diethyl ether and petroleum ether (1:1) mixture. Reaction products were analyzed by HPLC as explained in the section 2.2.6.4. Lycopene cyclase activity was calculated in terms of β-carotene formed and was expressed as nM of β- carotene formed mg protein^{-1} h^{-1}.

4.2.2.2. Carotenoid content of H. pluvialis cells and cell fractions

Cell fractions were prepared as described in the section 4.2.2.1.2. Carotenoid extraction and analyses were done as explained in section 2.2.6.

4.2.2.3. Qualitative analysis of proteins at different stages of H. pluvialis

Ten ml of *H. pluvialis* culture grown to different stages were centrifuged and pellets were suspended in 2 M NaOH for 1 h in ice as described by Whitelam and Codd (1982) and the alkaline solubilized protein was determined by Lowry's method (Lowry et al. 1951) using bovine serum albumin as the standard. The protein content was expressed in mg per litre of culture.

4.2.2.4. Antioxidant enzyme activities

4.2.2.4.1. Preparation of extracts for enzyme assays

Cells were harvested at 1,400 × g for 5 min and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1% PVP, 0.25% Triton X-100 and 1 mM PMSF for SOD analysis. For APX analysis extraction buffer also contained 0.5 mM ascorbate. The cells were ground with a mortar and pestle under liquid nitrogen. The suspensions were centrifuged again for 20 min at 4°C and supernatants were used for enzyme assays.

4.2.2.4.2. Enzyme assays

SOD (EC 1.15.1.1) was measured spectrophotometrically, based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan. The reaction mixture contains 0.5 ml of enzyme extract, 1 ml of 50 mM sodium carbonate, 0.4 ml of 24 μM NBT and 0.2 ml of EDTA. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 30 min at 25°C. Control was simultaneously run without enzyme extract. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT to 50%. The specific activity was

expressed in terms of units per mg protein (Murthy et al. 2002). APX (EC 1.11.1.11) activity was assayed according to Choo et al. (2004). Briefly, the reaction mixture consisted of 100 µl sample extract and 50 mM phosphate buffer (pH 7.0) containing 0.5 mM ascorbic acid (ASC) and 0.1 mM H_2O_2 in a total volume of 1 ml. The consumption of ASC by APX was measured by the decrease in absorbance at 290 nm (ε = 2.8 mM cm^{-1}) at 25°C.

4.2.3. Influence of CO_2 enrichment on carotenoid accumulation

4.2.3.1. Algal culture growth

One week old green vegetative cells were harvested by centrifugation at 1,400 × g for 5 min and resuspended either in complete medium (Bold's Basal medium) or in nutrient limiting medium (similar to complete medium except that it contains $1/10^{th}$ of N and P). Forty five ml of media (complete medium or nutrient limiting medium) was inoculated with 5 ml of stock culture in respective media in the upper chamber of two tier flask. Initial cell density was adjusted to 15×10^4 cells ml^{-1} by counting cell number using haemacytometer. Then cultures were kept in 250 ml normal Erlenmeyer flask or two-tier flask (Usha et al. 2001). 100 ml of 3M potassium carbonate buffer ($KHCO_3$-73 ml plus K_2CO_3-27 ml) was added to lower chamber of two-tier flask to generate 2% CO_2 for CO_2 supplementation treatments, and for other treatments lower chamber was filled with 100 ml of sterile water. Later, cultures were added with NaCl 17.1 mM plus SA 4.4 mM or without any addition, and exposed to continuous high light intensity of 60 µmol m^{-2} s^{-1} at 25 ± 1°C for 10 days. All the treatments were carried out in triplicate.

4.2.3.2. Growth and pigment analyses

Algal cell number was determined by counting algal cells using Neubauer haemacytometer. Known volume of culture was centrifuged at 3000 × *g* for 10 min and dry weight of the biomass was estimated as described in section 2.2.5.2. An aliquot of culture was harvested and freeze dried. Pigment extraction and analyses were carried out as per the procedure given in section 2.2.6.

4.2.4. Influence of salicylic acid and methyl jasmonate on antioxidant systems of *H. pluvialis*

4.2.4.1. Culture conditions

One week old green vegetative cells were transferred to either low light (30 µmol m^{-2} s^{-1}) or high light intensity (60 µmol m^{-2} s^{-1}) and different concentrations of ScA and MJ were added. 100mM ScA stock was prepared in 70% ethanol and 424 mM MJ stock in DMSO.

4.2.4.2. Growth and pigment analyses

Growth and pigment analyses were done as described in section 2.2.5 and 2.2.6.

4.2.4.3. Antioxidant enzyme activities

Six hours after exposure to required treatments cells were harvested. Enzyme extraction and assays for SOD and APX were done as explained in section 4.2.2.4. For CAT assay, enzyme extract preparation was similar to SOD. CAT (EC 1.11.1.6) activity was determined according to the method of Beers and Sizer (1952). The reaction mixture consisted of 100 µl sample extract and 50 mM phosphate buffer (pH 7.0) containing 18 mM H_2O_2 in a total volume of 3 ml. The consumption of H_2O_2 by CAT was measured by the decrease in absorbance at 240 nm (ε = 39.4 mM cm^{-1}) at 25°C.

4.2.5. Experimental design and data analysis

Each experiment was carried out with three replications. The data were analyzed by one way analysis of variance and post-hoc mean separations were performed by Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

4.3. Results

4.3.1. Differential display of light regulated transcripts in *H. pluvialis*

4.3.1.1. Pigment profile of H. pluvialis *under stress conditions*

Under stress condition for 2 days, reduction in chlorophyll, total carotenoids, β-carotene and lutein, and an increase in astaxanthin were observed (Table 4.3). Compared to NS condition, ND produced more astaxanthin, however due to rapid decrease in primary carotenoids, more reduction in total carotenoid was observed in ND cells compared to NS.

Table 4.3. Pigment contents (mg g^{-1} biomass) of *H. pluvialis* cells under stress conditions

Stress conditions *	Astaxanthin	β-carotene	Lutein	Chlorophyll
Green cells	0 ^{d}	3.35 ^{a}	34.30 ^{a}	37.25 ^{a}
NS/HL	1.28 ^{bc}	1.99 ^{cd}	32.47 ^{ab}	31.07 ^{b}
ND/HL	1.92 ^{b}	2.16 ^{c}	24.36 ^{bc}	22.73 ^{c}
ND/HL/NC	1.37 ^{bc}	1.55 ^{d}	19.48 ^{cd}	22.40 ^{c}
ND/HL/NA	3.22 ^{a}	2.50 ^{bc}	27.26 ^{b}	21.30 ^{c}
ND/HL/NC/NA	3.21 ^{a}	2.36 ^{bc}	25.57 ^{bc}	16.65 ^{cd}

Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).
* *H. pluvialis* cells were exposed to stress conditions for 2 days

4.3.1.2. Identification of differentially expressed transcripts under stress conditions

To elucidate the molecular processes occurring during carotenogenesis under stress condition, transcriptome analysis was undertaken using DD RT-PCR in *H. pluvialis*. *H. pluvialis* green vegetative motile cells and cells exposed to high light intensity (97 μmol m^{-2} s^{-1}) for two days were used to isolate RNA. First strands were synthesized using 7 anchored primers. For further amplification, several arbitrary primers were screened and five primers which gave clear bands were selected for further study. With 35 primer combinations, 109 bands that were differentially expressed under HL stress were identified (Figure 4.1).

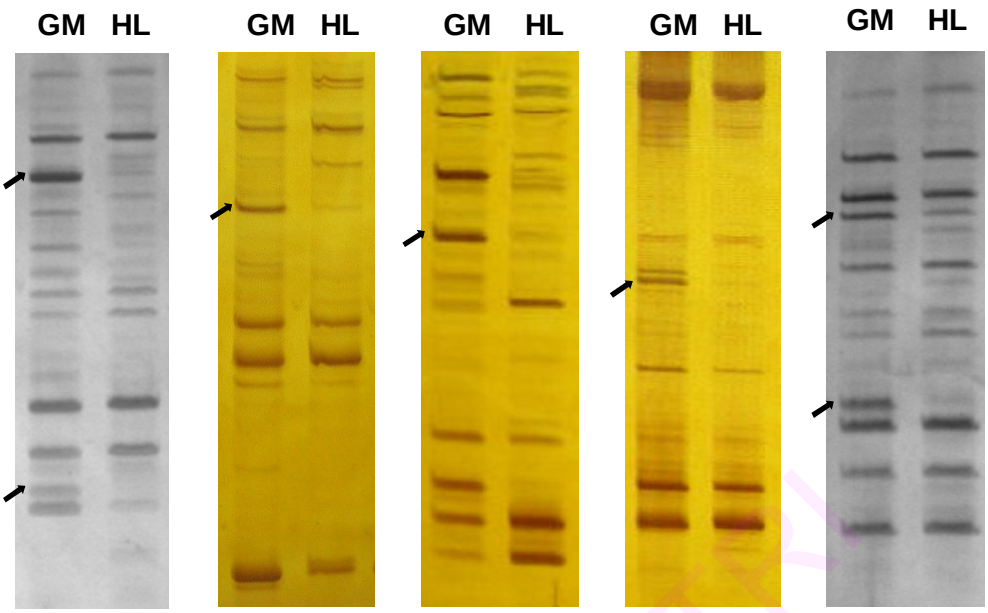


Figure 4.1. Differential display of mRNAs from green vegetative (GM) and high light (HL) exposed cells of *H. pluvialis* using primers combination of DD1/OPA20, DD3/OPD07, DD1/OPD07, DD2/OPD07 and DD3/OPA20. Arrows indicate differentially expressed cDNA bands that were excised for further analysis.

The cDNAs were eluted from the gel slices and reamplified using the same primer combination that generated it. From the 109 cDNAs, after removal of false positives, cDNA fragments were cloned into pKRX-T vector and six colonies per plate were picked randomly and subjected to colony PCR to verify the presence of the correct cDNA fragment. Because of the possibility of comigration associated DDRT-PCR (Zhang et al. 2005), 6 clones obtained from each band on the gel were examined. Finally 34 cloned cDNA fragments were sequenced, of which 14 being down regulated and 20 being up-regulated under HL stress as observed in the DD gel. Their nucleotide sequences were then analysed by searching for protein homologies against the GenBank database using the BLASTX program. For sequences which are having no significant homology to the available database, homology search was repeated using BLASTN program for nonredudant nucleotide sequences. The results were summarized in Table 4.4 and 4.5.

Table 4.4. Sequence homologies of the clones detected by Differential Display

Clone name	Expression	Fragment size (nt)	Best hits (BLASTX) analyses to nr database		
			Homology	Accn. No.	Score (E value)
Photosynthesis and carotenoid synthesis					
H53.1*	Down	208	Beta carotene C-4 oxygenase (*crt01*) gene, promoter region and 5' UTR *(Haematococcus pluvialis)*	AY 820775	114 (1e-22)
H64.1*	Down	205	Beta carotene C-4 oxygenase (*crt01*) gene, promoter region and 5' UTR *(Haematococcus pluvialis)*	AY 820775	114 (1e-22)
H97.1	Down	351	D1 reaction center protein of photosystem II *(Scenedesmus obliquus)*	YP 635976	212 (9e-54)
Metabolism					
H4.1*	Up	422	Putative polyhydroxyalkanoic acid system (*Salinibacter ruber)*	YP_446347	50.4 (4e-05)
H7.1*	Up	352	EPSP (5-enolpyruvylshikimate 3-phosphate) synthase mRNA *(Oryza sativa)*	AF 413082	42.8 (0.62)
H69.1*	Up	194	Cinnamoyl-CoA reductase mRNA, complete cds *(Arabidopsis thaliana)*	NM 106297	42.8 (0.53)
H69.2	Down	240	Ferrochelatase (*Rattus norvegicus)*	NP 001101904	34.3 (3.2)
H97.2	Down	178	Phospholipid/glycerol acyltransferase *(Cyanothece* sp.)	ZP 03145413	35.0 (1.9)
Transport/binding proteins and lipoproteins					
H13.2	Up	438	Ribose ABC transporter, ATP-binding protein (*Escherichia coli)*	ZP 03052513	276 (3e-73)
H52.2	Up	352	Transport transmembrane protein *(Ralstonia solanacearum)*	NP 522224	33.9 (4.3)
H107.1	Up	181	Putative lipoprotein (*Clostridium botulinum)*	ZP 02863168	52.8 (3e-06)
Stress related proteins					
H9.1	Down	331	LysR family transcriptional regulator *(Sphingomonas wittichii)*	YP 001263757	37.7 (0.29)
H80.1*	Up	99	Putative nucleotide binding site; leucine rich repeat resistance protein *(Pyrus communis)*	AJ581773	41.0 (0.98)
Genetic information processing					
H8.1	Up	515	Recombination activating protein 1 (*Tenrec ecaudatus)*	AAQ 57546	37.7 (0.35)
H31.1	Up	410	Putative reverse transcriptase *(Oryza sativa)*	AAR 89045	33.9 (4.2)
H55.1*	Up	197	Peptidyl-tRNA hydrolase 1 homolog (*S. cerevisiae*)	NM 001108580	42.8 (0.54)

*- analyzed by BLASTN

Table 4.5. Clones showing unknown and hypothetical proteins and no similarity detected by Differential Display

Clone name	Expre-ssion	Fragment size (nt)	Best hits (BLASTX) analyses to nr database		
			Homology	Accn. No.	Score (E value)
H2.1	Up	428	Orf122 (*Chlorobium tepidum)*	AF 287482	69.3 (9e-11)
H7.2	Up	707	Hypothetical protein (*Vitis vinifera)*	CAN 70790.1	135 (3e-30)
H13.1	Up	181	Hypothetical protein (*Chlamydomonas reinhardtii)*	XP 001698950	77.4 (3e-13)
H22.1	Up	146	Hypothetical protein (*Bacteroides capillosus*)	ZP 02038205	37.4 (0.39)
H22.2	Up	303	Unnamed protein product *(Kluyveromyces lactis)*	XP 453846	55.1 (5e-16)
H25.1	Down	155	Predicted protein (*Trichoplax adhaerens)*	XP 002118239	51.2 (3e-05)
H25.2	Down	245	Hypothetical protein *(Trichoplax adhaerens)*	XP 002118239	65.1 (2e-09)
H31.2	Up	179	Hypothetical protein (*Chlamydomonas reinhardtii*)	XP 001698950	75.9 (1e-12)
H32.1	Down	428	Unnamed protein product (*Kluyveromyces lactis)*	XP 453846	67.4 (1e-19)
H32.2	Down	180	Hypothetical protein (*Chlamydomonas reinhardtii)*	XP 001698950	77.4 (3e-13)
H32.3	Down	558	Hypothetical protein (*Aspergillus clavatus)*	XP 001269594	109 (1e-22)
H77.1	Up	267	Hypothetical protein (*Nematostella vectensis)*	XP 001618098	72.4 (6e-16)
H77.2	Up	122	ORF64d (*Pinus koraiensis)*	YP 001152204	38.9 (0.13)
H88.3	Down	363	GK18251 (*Drosophila willistoni*)	XP 002066362	35.0 (1.9)
H97.3	Down	428	Hypothetical protein (*Aspergillus clavatus)*	XP 001269594	95.5 (1e-18)
H109.1	Down	250	Hypothetical protein (*Bacillus megaterium)*	AAO 52807	92.8 (8e-18)
H34.1	Up	434	No significant similarity		
H80.2	Up	168	No significant similarity		

These differentially expressed transcripts include transcripts coding for proteins related to general metabolism, photosynthesis, carotenoid synthesis, lipid synthesis, tetrapyrrole synthesis, transporter proteins, defense signaling and genetic information processing (Table 4.4). About 17 transcripts were having homology to unknown and hypothetical proteins and 2 were having no significant similarity to available database (Table 4.5).

4.3.1.3. Confirmation of a set of high light stress regulated genes

To confirm the differentially expressed transcripts by DDRT-PCR, we investigated transcript levels of clone H97.1 on RNA isolated from green vegetative and high light stress-induced *H. pluvialis* cells by RNA dot blot and northern blot analysis (Figure 4.2).

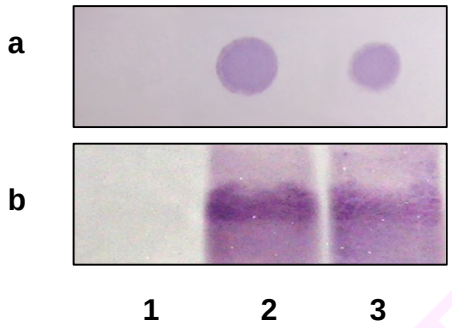


Figure 4.2. RNA dot blot (a) and northern blot (b) analysis of differentially expressed cDNA clone H97.1 isolated from differential display. Total RNAs of 0.1 µg from green vegetative cells (2) and cells exposed to HL for 2 days (3) were spotted on nylon membrane or fractionated on a 1.2% agarose gel containing 7% formaldehyde and blotted onto nylon membrane for hybridization. Lane 1 – water control.

The results showed down regulation of transcripts of this clone under high light stress condition. Further analysis of differentially expressed transcripts by RT-PCR for six representative cDNAs on RNA isolated from non-stressed and stress-induced (high light intensity 97 µmol m^{-2} s^{-1}, nutrient deficiency ($1/10^{th}$ of N and P), 17.1 mM NaCl, 4.4 mM SA and NaCl/SA) *H. pluvialis* cells revealed the level of mRNA expression under stress condition significantly varied from green vegetative cells, clearly demonstrating the reliability of the mRNA expression data obtained by DD RT-PCR.

4.3.1.4. Differential expression of stress regulated genes

Expression analysis showed 4 clones were up-regulated and 2 were down-regulated by HL stress. Clones up-regulated by HL include H-7.1, H-8.1, H-13.2 and H-69.1 coding 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, recombination activating protein 1, ribose ABC transporter ATP-binding protein and cinnamoyl-CoA reductase respectively.

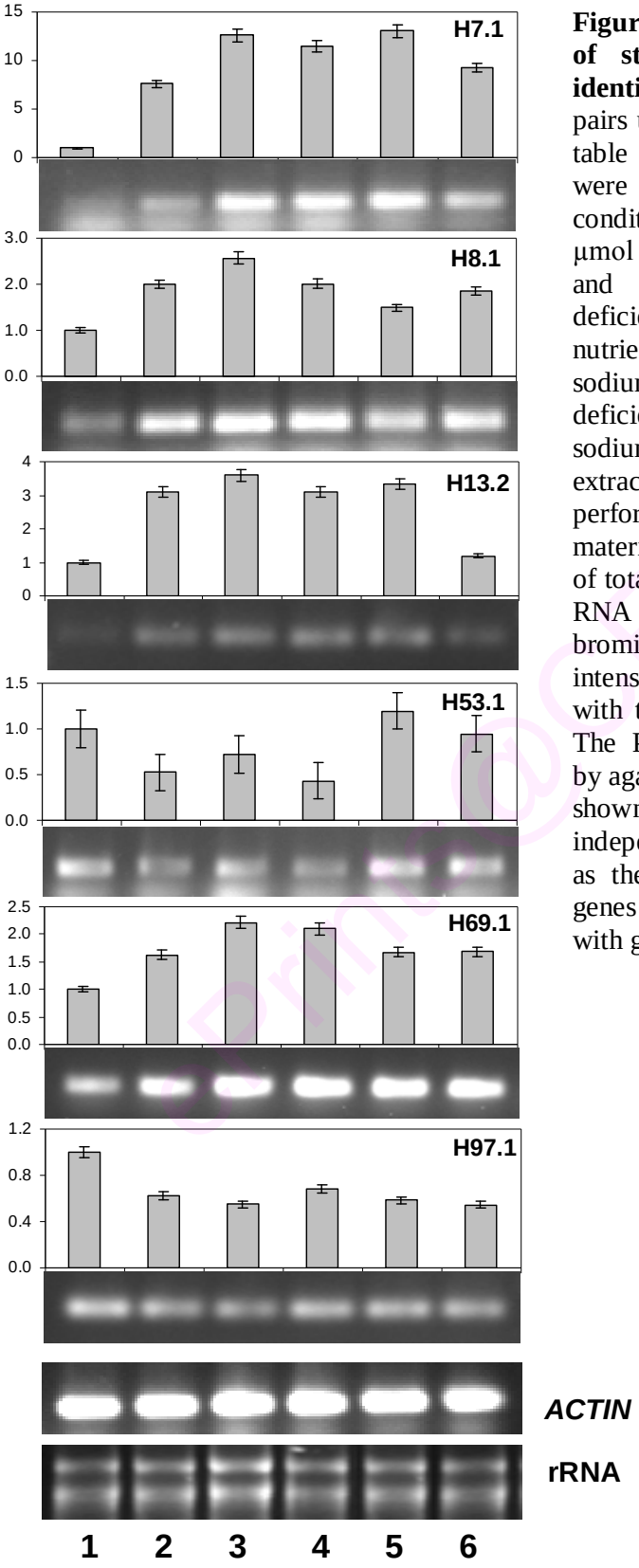


Figure 4.3. Differential expression of stress regulated transcripts identified by DD RT-PCR. Primer pairs used for RT-PCR are listed in table 4.2. Green motile cells (1) were exposed to different stress conditions - high light intensity 97 µmol m^{-2} s^{-1} (2) , nutrient deficiency and high light (3), nutrient deficiency, high light and NaCl (4), nutrient deficiency, high light and sodium acetate (5) and nutrient deficiency, high light, NaCl and sodium acetate (6) for 2 days. RNA extraction and RT-PCR were performed as described in the materials and methods with 0.1 µg of total RNA. For comparison, total RNA was stained with ethidium bromide (lower panel). The band intensity of each gene was adjusted with the band intensity of *ACTIN*. The PCR products were analysed by agarose gel electrophoresis. Data shown are mean±SD of three independent experiments expressed as the fold increase of respective genes expression in comparison with green motile vegetative cells.

The clone H7.1 showed 7.6-fold up-regulation upon exposure to HL, which was further enhanced by additional stresses such as ND, NC and NA with maximum transcript level of 13.0-fold upon ND/HL/SA exposure (Figure 4.3). The clone H8.1 coding recombination activating protein 1 was enhanced by HL (2-fold) and further increased by ND/HL exposure (2.6-fold) (Figure 4.3). Ribose ABC transporter ATP-binding protein coding clone H13.2 transcripts was also up-regulated by stress conditions with maximum transcript level of 3.6-fold by ND/HL. The clone H69.1 coding cinnamoyl-CoA reductase also showed enhanced transcript levels upon exposure to stress conditions with maximum of 2.2-fold by ND/HL (Figure 4.3).

The clone coding for 5'UTR of β-carotene oxygenase (H53.1) was down-regulated by HL which was further reduced by exposure to ND and NC condition. While addition of SA to ND condition enhanced the transcript levels to 1.2-fold and further addition of NC decreased the transcript levels to green vegetative cells (Figure 4.3). Another clone (H97.1) which is down regulated by HL stress codes for photosystem II reaction centre D1 protein, is partially cloned for the first time in *H. pluvialis*. Expression of this clone is down regulated by all stresses studied (Figure 4.3).

4.3.2. Biochemical changes associated with carotenogenesis in *H. pluvialis*

4.3.2.1. Carotenoid enzyme activities

Activities of lycopene cyclase and β–carotene ketolase, important enzymes in astaxanthin biosynthetic pathway, were studied and presented in table 4.6 and 4.7.

Table 4.6. Lycopene cyclase activity of *H. pluvialis* cells

Conditions	Lycopene cyclase activity (nM of β-carotene formed mg protein^{-1} h^{-1})
Green motile cells	110.6 [b]
Stress induced cells	456.5 [a]

Means followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$)

Lycopene cyclase activity estimated as nM of β-carotene formed mg protein^{-1} h^{-1} was increased under stress condition (Table 4.6). β-carotene ketolase activity (ng of ketocarotenoids formed mg protein^{-1} h^{-1}) showed no changes in the enzyme activity of chloroplast fraction under stress condition. While lipid fraction isolated only under

stress condition showed increased activity compared to chloroplast fraction (Table 4.7).

Table 4.7. β–carotene ketolase activity of *H. pluvialis* cells

Conditions	Fraction	β–carotene ketolase activity (ng of ketocarotenoids formed mg protein^{-1} h^{-1})
Green motile cells	chloroplast	606.5 [b]
Stress induced cells	chloroplast	606.9 [b]
Stress induced cells	lipid	2523.9 [a]

Means followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$)

4.3.2.2. Carotenoid content of H. pluvialis cells and cell fractions

In the present study, it was observed that green motile *H. pluvialis* cells did not yield lipid fraction when it was fractionated. Figure 4.4 showed that chloroplast fraction is having lutein, β-carotene and chlorophyll as pigments while lipid fraction is having β-carotene, astaxanthin, canthaxanthin and echinenone as pigments.

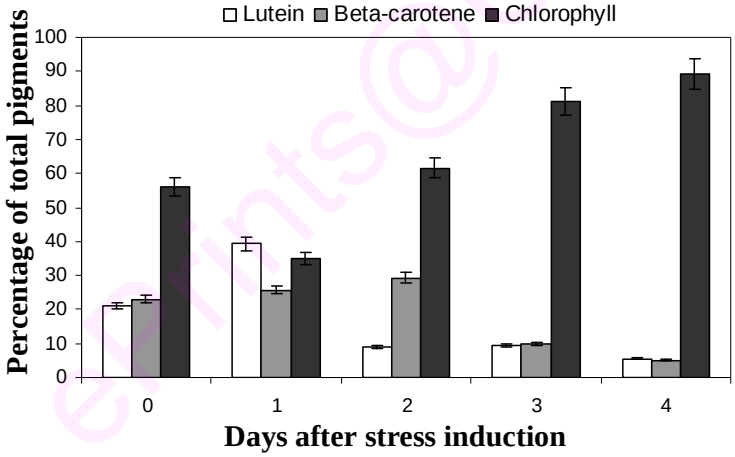


Figure 4.4. Pigment composition of chloroplast fraction of *H. pluvialis* cells

In the chloroplast fraction, proportion of chlorophyll increases and proportion of lutein and β-carotene decreases over a period of time (Figure 4.4). While in the lipid fraction, proportion of astaxanthin was increased, the proportion of β-carotene, canthaxanthin and echinenone was decreased (Figure 4.5).

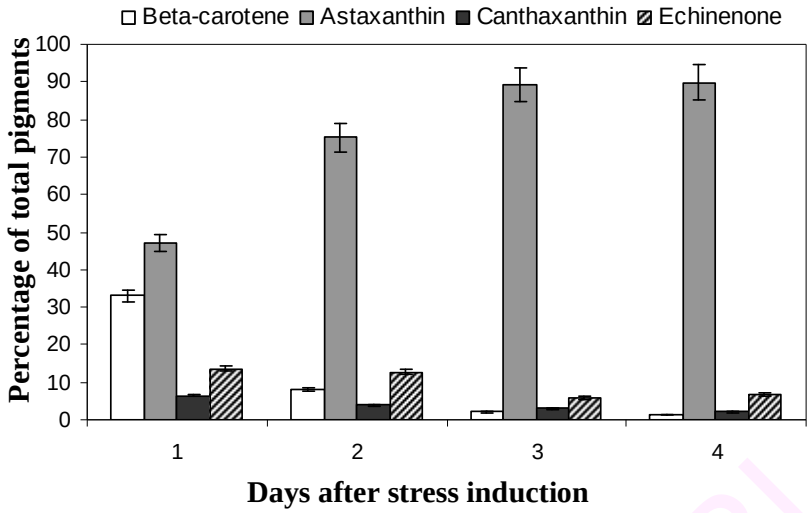


Figure 4.5. Pigment composition of lipid fraction of *H. pluvialis* cells

Changes in the composition of total astaxanthin over a period of time was studied and presented in Figure 4.6.

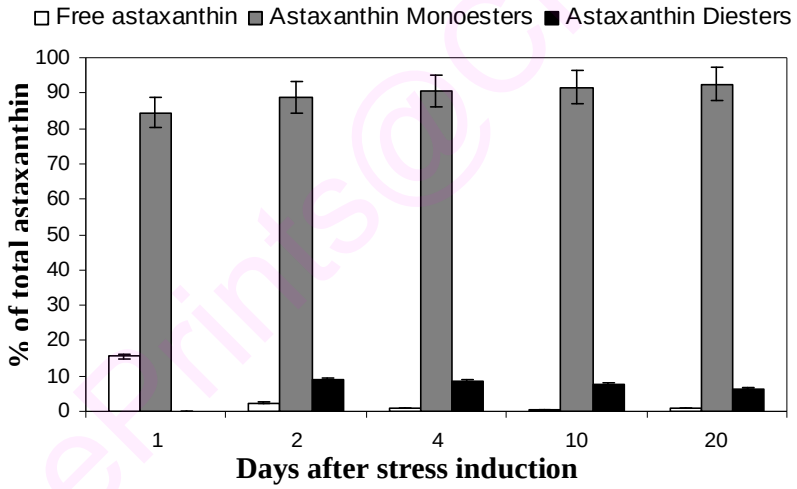


Figure 4.6. Composition of total astaxanthin of *H. pluvialis* cells

The results revealed that proportion of free astaxanthin decreases while proportion of astaxanthin mono- and diesters increases with astaxanthin monoesters constituting the maximum portion at any given time. And among different forms of astaxanthin, astaxanthin monoester alone represents 84-93% while astaxanthin diesters and free astaxanthin constituted about 0-9% and 0.6-16% of total astaxanthin respectively.

4.3.2.3. Qualitative analysis of proteins at different stages of H. pluvialis

Total protein content of *H. pluvialis* cells in a litre of NS and ND cultures exposed to HL over a period of time was estimated and presented in Figure 4.7. The results indicated that protein content showed decreasing trend in ND culture and increasing trend in NS culture.

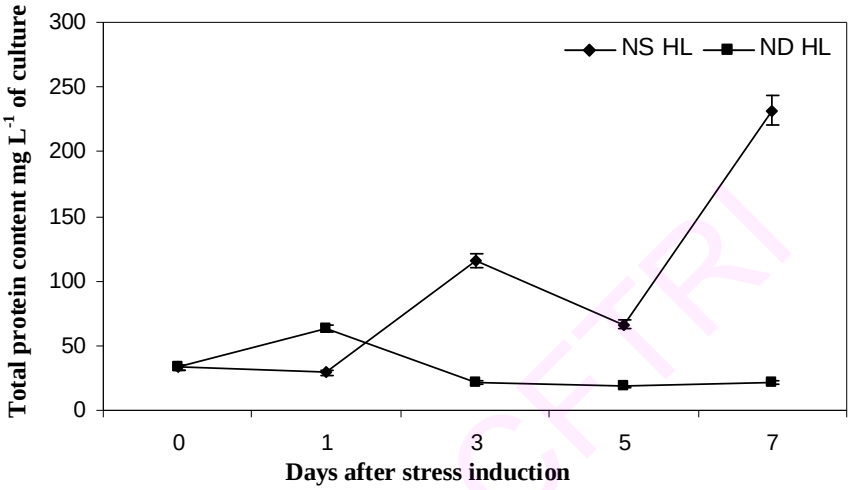


Figure 4.7. Total protein content of *H. pluvialis* cultures

4.3.2.4. Antioxidant enzyme activities

Activities of antioxidant enzymes such as APX and SOD of *H. pluvialis* cells cultured in NS and ND media and exposed to high light were studied and presented in Figures 4.8 and 4.9. Both APX and SOD showed decreased activities when cells were cultured in NS condition and increased activities in ND condition.

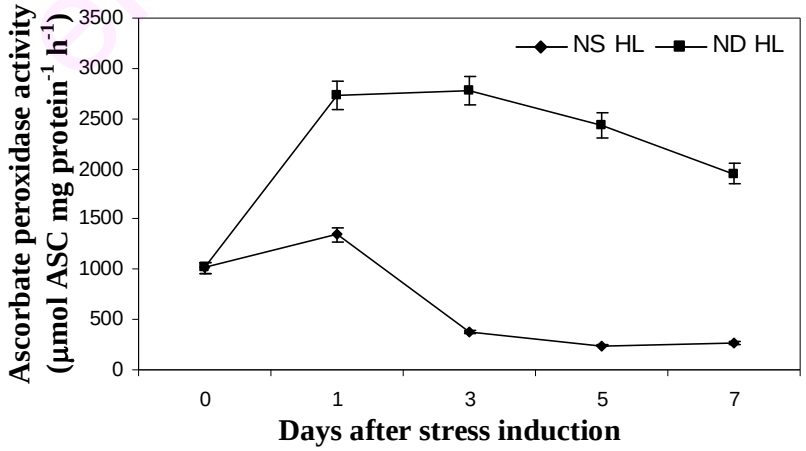


Figure 4.8. Ascorbic peroxidase activity of *H. pluvialis* cells

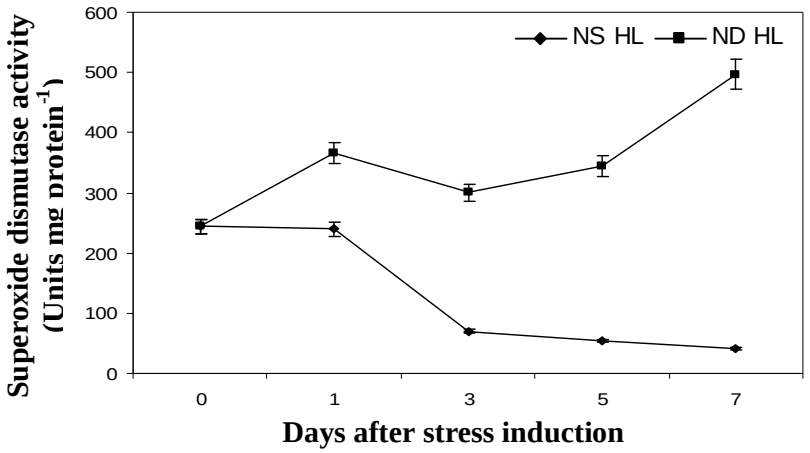
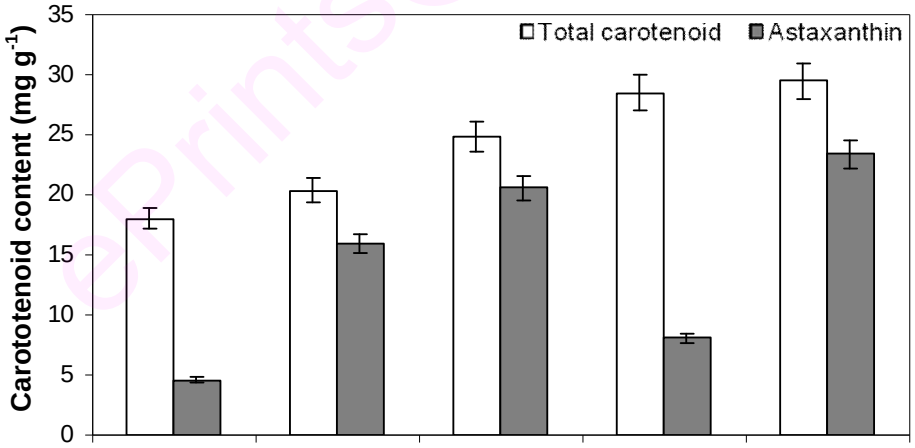


Figure 4.9. Superoxide dismutase activity of *H. pluvialis* cells

4.3.2.5. Influence of CO_2 enrichment on carotenoid accumulation

Influence of CO_2 enrichment along with NaCl addition was studied and presented in Figures 4.10, 4.11 and 4.12. Figure 4.10 showed that exposure of *H. pluvialis* cultures to HL and CO_2 environment with or without NaCl addition enhanced both total carotenoid and astaxanthin production. Though addition of NaCl alone to HL exposed cultures increased total carotenoid production, it decreased both astaxanthin content and biomass production drastically.



Stress	NS	ND	CO_2	NaCl	CO_2/NaCl
Biomass (mg L^{-1})	1318 [a]	659 [c]	782 [bc]	296 [d]	798 [bc]

Figure 4.10. Effect of CO_2 enrichment and salinity on growth and carotenoid production of *H. pluvialis* under high light condition for 10 days. Means followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

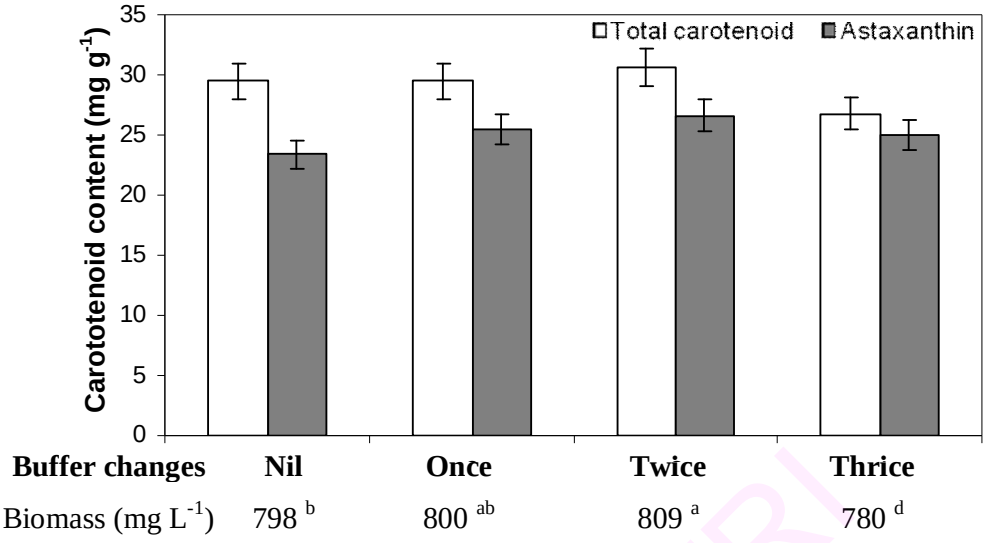


Figure 4.11. Effect of CO_2 enrichment on growth and carotenoid production of *H. pluvialis* under high light and salinity stress for 10 days. Means followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

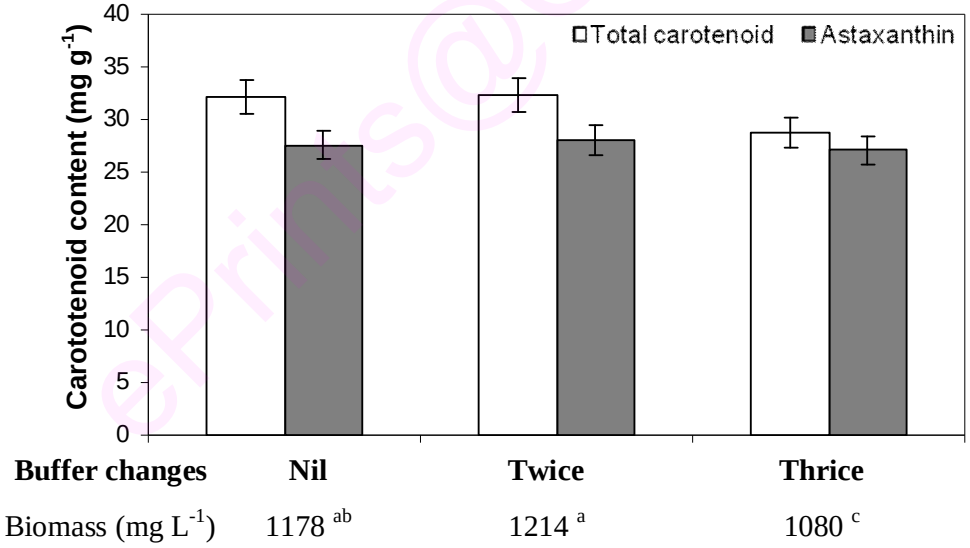


Figure 4.12. Effect of CO_2 enrichment on growth and carotenoid production of *H. pluvialis* under high light and salinity stress for 20 days. Means followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$)

From the Figure 4.11, it can be concluded that changing the carbonate buffer in the lower compartment of 2-tier vessel to provide constant CO_2 environment was found to be efficient in enhancing carotenoid and astaxanthin content along with biomass production (Figure 4.11). Though changing the carbonate buffer more than

twice affected biomass, carotenoid and astaxanthin production (Figure 4.11). Similar trend was observed when *H. pluvialis* cultures were exposed to stress conditions for 20 days (Figure 4.12).

4.3.2.6. Influence of salicylic acid (ScA) and methyl jasmonate (MJ) on antioxidant systems of H. pluvialis

4.3.2.6.1. Effect of ScA and MJ on the growth and encystment

To study the influence of ScA and MJ on *H. pluvialis*, cultures were exposed to different concentrations (10-500 µM) of ScA and MJ incubated in either low (30 µmol m^{-2} s^{-1}) or high (60 µmol m^{-2} s^{-1}) light intensities for 8 days. At the time of ScA and MJ addition, number of cells in the culture was 32.5×10^4 cells per ml and among them 1.0×10^4 cells per ml were cysts.

ScA treatment increased the biomass in concentration dependent manner (up to 100 µM) irrespective of the light condition used (Table 4.8). ScA at 500 µM concentration reduced the biomass under low light and resulted in bleaching of cells under high light. Similar trend was observed for cell count (Table 4.8). Lowest concentration of MJ (10 µM) produced comparatively high DW both under low and high light conditions (Table 4.8). However, the biomass decreased with increased MJ concentration and reached below that of the control at 500 µM concentration. Similar trend was observed for cell number also and 100 µM of MJ produced maximum number of cells under high light conditions. Growth inhibition by high concentration of MJ is not pronounced like ScA. Both ScA and MJ, increased the % of cyst cells formed under low light, whereas under high light, they reversed the effect of high light stress by decreasing the % of cyst cells formed.

Table 4.8. Effects of salicylic acid (ScA) and methyl jasmonate (MJ) on growth and encystment of *H. pluvialis*

Conditions	Concentration (μM)	No. of cells ($\times 10^4$ cells/ml)	% of cysts	Dry wt g L^{-1}
ScA LL	**0**	83 ^{cd}	6 ^{d}	0.45 ^{bc}
	10	81 ^{cd}	6 ^{d}	0.52 ^{b}
	50	85 ^{cd}	7 ^{d}	0.55 ^{b}
	100	90 ^{c}	8 ^{d}	0.59 ^{ab}
	500	43 ^{e}	94 ^{a}	0.26 ^{cd}
ScA HL	**0**	96 ^{c}	63 ^{b}	0.65 ^{a}
	10	99 ^{c}	37 ^{c}	0.67 ^{a}
	50	104 ^{bc}	36 ^{c}	0.67 ^{a}
	100	109 ^{bc}	35 ^{c}	0.67 ^{a}
	500	Cells bleached		0.10 ^{e}
MJ LL	**0**	83 ^{cd}	6 ^{d}	0.45 ^{bc}
	10	98 ^{c}	3 ^{d}	0.47 ^{b}
	50	85 ^{cd}	7 ^{d}	0.45 ^{bc}
	100	71 ^{d}	11 ^{cd}	0.41 ^{bc}
	500	42 ^{ef}	40 ^{bc}	0.35 ^{c}
MJ HL	**0**	96 ^{c}	63 ^{b}	0.65 ^{a}
	10	101 ^{bc}	39 ^{bc}	0.68 ^{a}
	50	125 ^{ab}	25 ^{c}	0.66 ^{a}
	100	142 ^{a}	11 ^{cd}	0.62 ^{ab}
	500	66 ^{d}	98 ^{a}	0.54 ^{b}

Data are presented as the mean of two separate experiments, each with three replicates. Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

4.3.2.6.2. Effect of ScA and MJ on pigments

The initial chlorophyll and carotenoid content of green vegetative cells were 35 and 12 mg g^{-1} DW respectively with lutein and β-carotene as major carotenoids, followed by neoxanthin and violaxanthin occurring in traces, with no secondary carotenoids like astaxanthin, canthaxanthin or echinenone. To study the effect of phytohormones on pigment production, green vegetative cells were treated with different concentrations of ScA and MJ and incubated under low light and high light for 8 days. Increasing concentrations of ScA reduced the chlorophyll content both under low and high light intensity (Figure 4.13).

MJ treatment increased the chlorophyll content under low light condition. But high light increased chlorophyll content upto 100 µM of MJ. Further increase in concentration of MJ reduced the chlorophyll content but still the content was higher than the control. Total carotenoid content was reduced by ScA irrespective of light intensity. MJ treatment has shown marginal increase in carotenoid content under low and high light conditions with significant increase at 500 µM concentration with the content of 48.19 and 35.21 mg g^{-1} respectively (Figure 4.13). HPLC analysis showed the presence of traces of neoxanthin, violaxanthin, canthaxanthin and echinenone in all treatments (Figure 4.14).

Though exposure of cells to ScA and low light decreased the total carotenoid content (Figure 4.13), it increased the proportion of astaxanthin which was 6.8-fold higher than control at 100 µM ScA (Figure 4.15). Higher concentration of ScA (500 µM) inhibited astaxanthin accumulation completely. ScA treatment at high light decreased the proportion of astaxanthin compared to control. MJ at 10 µM increased astaxanthin % both under low and high light conditions but further increase in MJ concentration decreased the % of astaxanthin. Both ScA and MJ caused overall increase in β-carotene under low light while high light exposure resulted in decreased β-carotene % (Figure 4.15). Lutein % was decreased at low concentrations of ScA (10 to 100 µM) under low light and 500 µM of ScA increased lutein % which is same as control. Exposure of cells to MJ under low light did not change lutein %. Both ScA and MJ at high light increased the lutein % (Figure 4.15).

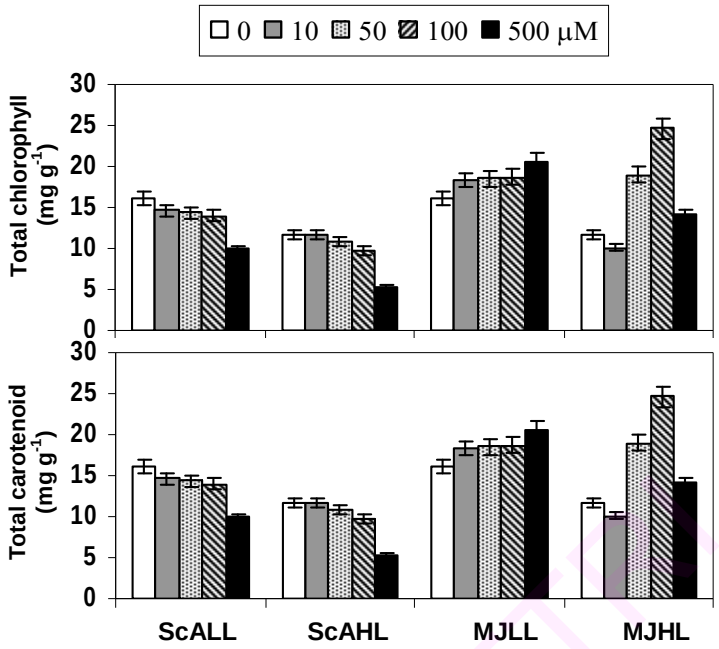


Figure 4.13. Effects of salicylic acid and methyl jasmonate on pigment contents of *H. pluvialis* under low light (LL) and high light (HL) intensity. Bars represent ± SE of the mean.

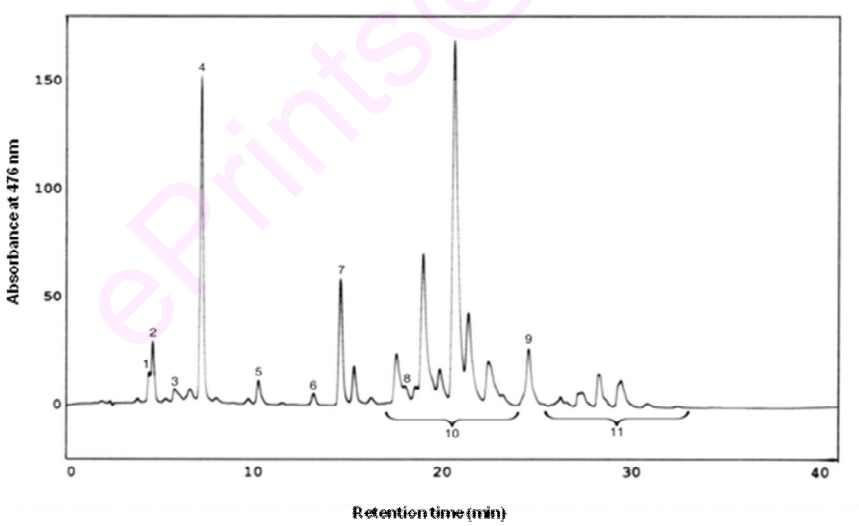


Figure 4.14. HPLC separation of carotenoids extracted from *H. pluvialis* cells exposed to high light for 8 days. Peaks were identified as (1) neoxanthin, (2) violaxanthin, (3) free astaxanthin, (4) lutein, (5) canthaxanthin, (6) chlorophyll b, (7) chlorophyll b', (8) chlorophyll a + echinenone, (9) β-carotene, (10) astaxanthin monoesters and (11) astaxanthin diesters.

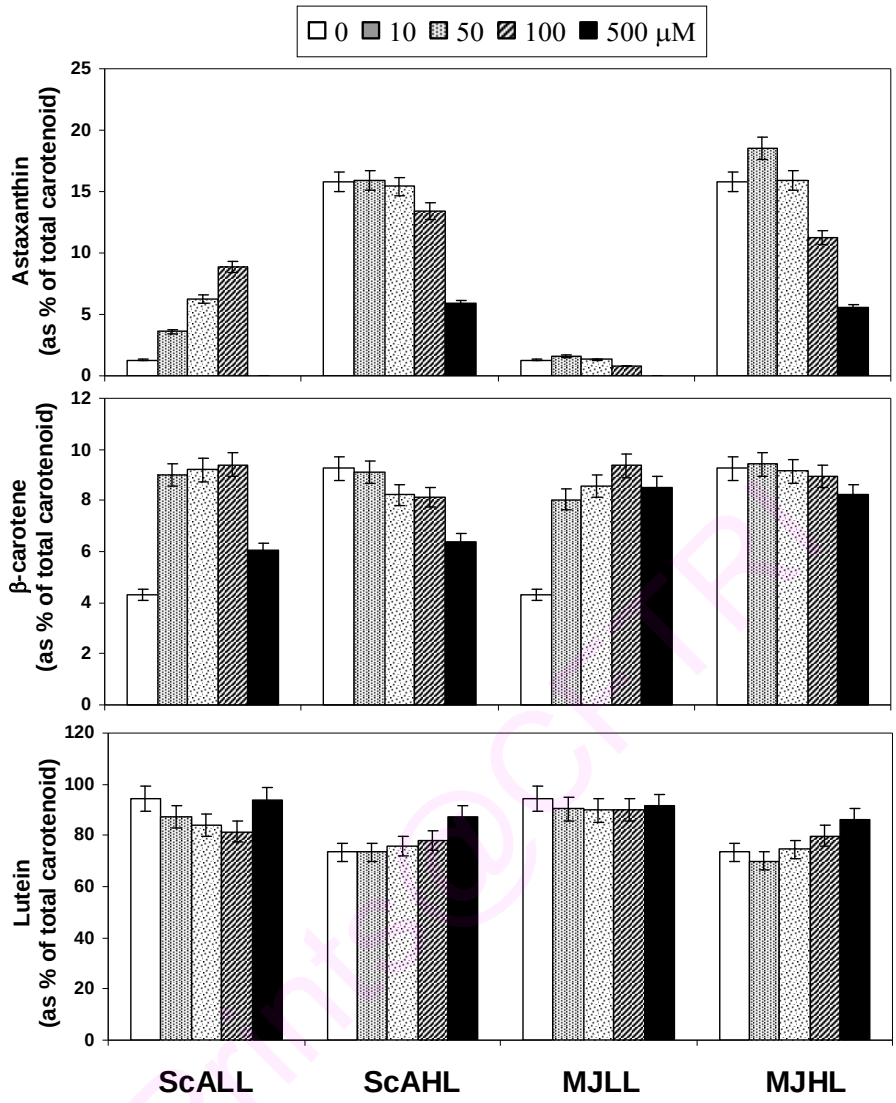


Figure 4.15. Effects of salicylic acid and methyl jasmonate on composition of carotenoids of *H. pluvialis* under low light (LL) and high light (HL) intensity. Bars represent ± SE of the mean.

4.3.2.6.3. Effect of ScA and MJ on the activities of antioxidant enzymes

Alterations in the activities of antioxidant enzymes, SOD, CAT and APX were studied in *H. pluvialis* cultures after exposure to ScA and MJ for 6 h. Increased SOD and APX activities and decreased CAT activities were observed in ScA treated low light exposed cells (Figure 4.16). Maximum activity of SOD and APX was observed for 500 µM of ScA, which were 4.5- and 15.5-fold higher respectively compared to control. In ScA treated high light exposed cultures, increase in SOD and CAT activities were observed in a concentration dependent manner with 500 µM of ScA treated cultures showing 3.3- and 1.2-fold respectively higher activity than control.

Under high light slight increase in APX activity was observed at lower concentrations of ScA and 500 µM of ScA produced 7.1-fold higher activity of APX (Figure 4.16).

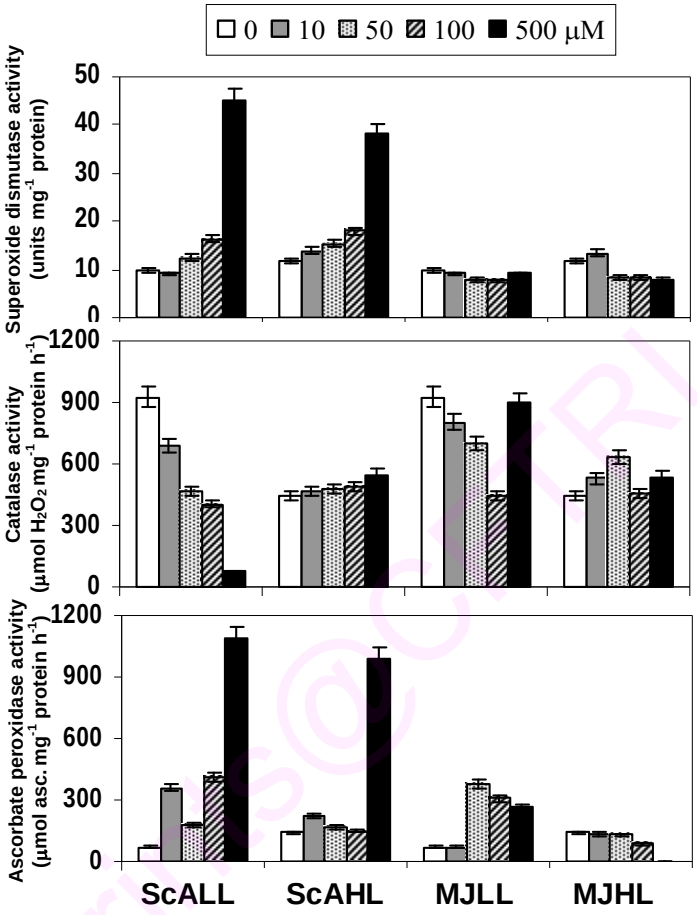


Figure 4.16. Salicylic acid and methyl jasmonate induced changes in activities of superoxide dismutase, catalase and ascorbate peroxidase in *H. pluvialis* cells exposed to low light (LL) and high light (HL) for 6 hours. Bars represent ± SE of the mean.

Under low light, SOD and CAT activities exhibited decrease in activities with increase in MJ concentration up to 100 µM. MJ treated low light exposed cultures have shown increased APX activities with maximum activity (5.4-fold higher than control) being observed at 50 µM of MJ (Figure 4.16). Under high light exposed conditions, MJ treatment reduced the SOD activity. Activity of CAT showed an increasing trend till 50 µM of MJ under high light (1.4-fold higher than control). APX activity decreased in a dose-dependent manner and complete inhibition was observed at 500 µM of MJ under high light (Figure 4.16).

4.4. Discussion

4.4.1. Differential display of light regulated transcripts in *H. pluvialis*

DD technique has been used to identify genes differentially expressed upon HL exposure of *H. pluvialis* cells. Knowledge of the functions of such genes will lead to better understanding of molecular mechanisms of astaxanthin biosynthesis and photo-protection. In the present study we have used DD technique since it can identify weakly expressed transcripts. Unlike earlier studies in *H. pluvialis*, in this study high light alone was used to identify differentially expressed genes to exclude the effects of other stress conditions.

Of the 109 differentially expressed transcripts obtained using 35 primer combinations, false positives were removed after re-amplification. About 34 clones have been sequenced and analysed in silico. Among the differentially expressed transcripts, three were having homology for photosynthesis and carotenoid synthesis related proteins. The clone H97.1 is having homology for D1 reaction center protein of photosystem II. Clone H97.1 is the first isolated *H. pluvialis* transcript that had homology to *psab* gene coding members of photosystem II reaction center protein D1. The decreased expression of this clone under high light stress (Table 4.4) is similar to the results obtained from other green algae and higher plants (Alfonso et al. 2000) and correlated with reduction in the amount of D1 protein of photosystem II during initial stages of exposure of *H. pluvialis* cells to stress (Wang et al. 2003b). Later reduction in D1 protein level was prevented by deposition of astaxanthin thus enabling the cell to maintain PS I function and structural integrity (Wang et al. 2003b). Expression analysis of H97.1 by RNA dot blot and northern blot analysis on RNA samples from green vegetative cells and high light stressed cells exhibited down regulation of H97.1 under high light stress, suggested reliability of DD RT-PCR for mRNA expression data. The reduction in expression of H97.1 transcript under various stress conditions (Figure 4.3) is correlated well with the reduction in photosynthetic pigments, chlorophyll, β-carotene and lutein (Table 4.3). Two clones, H34.1 and H53.1 down regulated by high light stress (Table 4.4) showed no significant homology when analyased by BLASTX but were found similarity with 5'UTR of *H. pluvialis* β-carotene oxygenase (*crt*01) (synonym: β-carotene ketolase) when analysed by BLASTN. Further analysis of H53.1 by RT-PCR showed that reduced expression of

this high light regulated transcript could be recovered and up-regulated when cells were exposed to HL/ND/SA (Figure 3.3). Though there are reports of up-regulation of β-carotene oxygenase under stress (Sun et al. 1998; Grünewald et al. 2000; Steinbrenner and Linden 2003), multiple forms of this gene with each responding differently to different stress conditions is reported (Huang et al. 2006a). Differential expression of this clone (Figure 4.3) is correlated well with the production of astaxanthin under stress condition (Table 4.3). The result from the present study suggested that cultural parameters and stress factors could be manipulated to achieve maximum astaxanthin production.

Five differentially expressed clones were identified to be involved in metabolism. Among them, three clones such as H97.2, H4.1 and H69.2 were down regulated while clones H69.1 and H7.1 having similarity to cinnamoyl-CoA reductase and EPSP synthase were up-regulated under high light stress (Table 4.4). EPSP synthase catalyses the sixth step of the shikimate pathway that is responsible for synthesizing aromatic compounds (Julio et al. 2006). The clone H69.2 was having similarity to ferrochelatase. Ferrochelatase catalyses crucial branch point of the tetrapyrrole synthesis pathway, insertion of Fe^{2+} into the tetrapyrrole ring of protoporphyrin IX to generate protohaem (Miyamoto et al. 1994). The phytochrome chromophore phytochrobilin is synthesized from haem (Gray 2003). Both H69.1 and H7.1 showed enhanced expression under all stress conditions studied (Figure 4.3).

Transcripts which are having homology to transport proteins identified from the present study were ribose ABC transport ATP-binding protein and transport transmembrane protein (Table 4.4). And both were up-regulated under HL stress (Table 4.4). Further analysis showed clone H13.2 was further up-regulated by additional stresses (Figure 4.3). In *E. coli* ribose ABC transport ATP-binding protein is involved in transporting D-ribose sugar, so that D-ribose can be used as a sole carbon source (Oh et al. 1999). In *H. pluvialis*, carotenoids are produced through methylerythritol phosphate (MEP) pathway where IPP, precursor for carotenoids is produced from pentose sugars (Lichtenthaler 1999; Hagen and Grünewald 2000). Moreover in dark grown *Chlorella zofingiensis* cells, various sugars have reported to be differentially regulating the transcription of the carotenoid biosynthetic genes. Glucose and mannose, which supported the best growth and astaxanthin production of the algal cells, also resulted in the highest transcript levels of all the genes (Sun et al.

2008). The gene β-carotene oxygenase have been found to be transiently up-regulated upon glucose treatment (Huang et al. 2006b) and the transcription of β-carotene oxygenase and β-carotene hydroxylase are differently regulated by the metabolism of glucose, through which the biosynthesis of astaxanthin is regulated (Li et al. 2008a).

Clone H9.1, having similarity for defense signaling molecule, LysR family transcriptional regulator (LTTR), was down regulated under high light stress in *H. pluvialis*. The LysR family is composed of more than 50 similar-sized, autoregulatory transcriptional regulators (LTTRs) and are present in diverse bacterial genera, archaea and algal chloroplasts (Schell 1993). In response to different coinducers, LTTRs activate divergent transcription of linked target genes or unlinked regulons encoding extremely diverse functions. They activate the transcription of operons and regulons involved in extremely diverse cellular functions including nitrogen fixation, oxidative stress response and bacterial virulence (Schell 1993). Most LTTRs, while activating expression of target genes, repress their own expression, frequently by the use of divergent promoters (Zaim and Kierzek 2003).

Two clones – H8.1 and H31.1 were identified to be involved in genetic information processing and were up-regulated under high light stress (Table 4.4). The clone H8.1 is coding for recombination activating protein 1 and clone H31.1 is coding for reverse transcriptase. Transcripts of H8.1 were up-regulated by different stress conditions (Figure 4.3). The high light induced clone H80.1 is having similarity for nucleotide binding site (NBS)-leucine rich repeat (LRR) resistance protein. The NBS-LRR gene family accounts for the largest number of known disease resistance genes, and is one of the largest gene families in plant genomes (Ameline-Torregrosa et al. 2008). Two clones H55.1 and H107.1 were having similarity for peptidyl-tRNA hydrolase 1 and lipoprotein respectively and were up-regulated under high light stress. In lipoprotein, the lipids or their derivatives may be covalently or non-covalently bound to the proteins. Many enzymes, transporters, structural proteins, antigens, adhesins and toxins are lipoproteins. Examples include the high density and low density lipoproteins which enable fats to be carried in the blood stream, the transmembrane proteins of the mitochondria and the chloroplast, and bacterial lipoproteins (Garrett and Grisham 1995). Seventeen clones were having homology to unknown and hypothetical proteins. There were two clones which had no significant homology in the database (Table 4.5). It is possible that these cDNAs may encode potentially novel proteins or belong to 5' or 3' specific ends of some known genes.

This analysis indicated that response to high light stress is a complex process, involving differential expression of functionally different groups of genes. A partial characterization of light regulated transcripts of *H. pluvialis* can be helpful for better understanding of regulation of carotenogenesis. Identification of these stress-regulated transcripts is an initial step towards cloning and characterization of full-length cDNAs and promoter regions. Such studies should identify common and/or unique regulatory elements and, thus, provide insight into the mechanism of a gene's individual expression, as well as its potential role in stress response. This information will in turn help us to understand better signaling and interactions between the major stress-response pathways.

4.4.2. Biochemical changes associated with carotenogenesis in *H. pluvialis*

The increased LCY activity under stress condition (Table 4.6) is similar to the observation of Usha (2000). It suggested increased precursor requirement for increased production of astaxanthin under stress condition. The observation of increased BKT activity in lipid fraction compared to chloroplast fraction is in line with the observation of Grünewald et al. (2001), confirming that BKT enzyme is more active in lipid vesicles compared to chloroplast.

Analysis of carotenoids of chloroplast and lipid fractions (Figures 4.4 and 4.5) revealed the presence of β-carotene in lipid globules. This substantiates the hypothesis that β-carotene might be exported from chloroplast to lipid globules (Grünewald and Hagen 2001) where it may be converted into canthaxanthin, echinenone and astaxanthin. Studies on composition of total astaxanthin revealed that proportion of free astaxanthin is decreased as stress progressed. This suggested that immediately after synthesis free astaxanthin is converted into mono- and diesters.

Increased antioxidant enzyme activities observed under ND condition (Figures 4.8 and 4.9) suggested that nutrient deficiency imposes an additional stress on the organism, so that more antioxidant enzymes along with astaxanthin are produced to protect the organism. Similar to the present study increased SOD activity was observed during Cu induced oxidative stress in *Chlorella vulgaris* (Mallick 2004).

4.4.3. CO_2 enrichment

In the present study it was observed that CO_2 enrichment with or without salinity stress enhanced carotenoid and astaxanthin production. Similar to this, enhanced

growth and carotenoid contents in *H. pluvialis*, *Chlorella vulgaris* and *Scenedesmus obliquus* wer observed under CO_2 supplemented condition (Usha et al. 2001). The increase in astaxanthin content by CO_2 enrichment (Figure 4.10) may be due to increased C/N ratio as suggested by Kakizono et al. (1992). The reduction in biomass and astaxanthin content by more times of buffer change may be induced by the toxic effect of more amount of CO_2 generated on biomass and astaxanthin content. Reduced biomass in NaCl added cultures showed the increased susceptibility of *H. pluvialis* cells to salinity under ND conditions.

4.4.4. Influence of salicylic acid and methyl jasmonate on antioxidant systems of *H. pluvialis*

ScA and MJ are naturally occurring signal molecules, and are involved in plant response to several environmental stress factors and reported to affect growth and development of plants. Similar to the present study Fung (2005) reported decreased biomass in *Chlorella zofingiensis*, when heterotrophically dark grown cells were exposed to 10^{-3} M of ScA, and further increase in ScA concentration resulted in bleaching of cells and cell death. ABA is reported to have morphogenetic effect on the cells of *H. pluvialis*, stimulating their transition to cyst formation (Kobayashi et al. 1997c). The decrease in cell number was also observed in *C. vulgaris* with JA treatment (Czerpak et al. 2006).

On contrary to the observation of the present study, ScA at 10^{-4} M had stimulating effect on chlorophylls and carotenoids in *Chlorella vulgaris* and at 10^{-6} M it showed slight decrease in carotenoid content (Czerpak et al. 2002). In *Arabidopsis thaliana* leaves, 1 mM ScA increased violaxanthin, neoxanthin, antheraxanthin, zeaxanthin and β-carotene, and further increase in ScA concentration to 5 mM, decreased the levels of these pigments (Rao et al. 1997). But in the present study, increased levels of astaxanthin and β-carotene was observed in low light ScA treated cells and decreased levels of these pigments were observed under high light (Figure 4.15). Jasmonic acid over the concentration range of 10^{-5} to 10^{-4} M inhibited chlorophylls and carotenoids in *Chlorella vulgaris* (Czerpak et al. 2006). Decreased chlorophyll, carotenoid, neoxanthin, violaxanthin, lutein and β-carotene were observed in MJ treated *Arabidopsis thaliana* (Jung 2004). But the present study showed an increase in chlorophyll, carotenoid and β-carotene in MJ treated low light exposed *H. pluvialis* cells (Figures 4.13 and 4.14). It is evident from the results that

response to ScA and MJ varies with organisms, concentration of phytohormones and light intensity.

The higher concentration of ScA caused a permanent change at the level of membrane organization of the cells that proved injurious for plant metabolism and growth (Uzunova and Popova 2000). This may be the possible reason for reduced growth and bleaching of cells at higher concentrations of ScA observed in the present study.

Treatment with signaling molecules may induce H_2O_2 production, which in turn may induce the synthesis or activate various transcription factors, which are associated with the induction of different antioxidant enzymes (Agarwal et al. 2005). Photooxidative stress also triggers other components of the dissipative process, enzymatic antioxidants. Various antioxidant enzymes appear to play an essential part of defense mechanisms against MJ and ScA induced oxidative stress. MJ treated cultures were having more carotenoid and chlorophyll while ScA treated cultures were having more antioxidant enzyme activities. The increase in ScA and MJ concentration may induce oxidative stress, which in turn may develop different defense mechanisms in the organism. The response to ScA and MJ may vary with organisms, concentration of phytohormones and light intensity. In addition, ScA and MJ may be acting as a stress modulator by changing the stress response of *H. pluvialis* to high light stress.

Both phytohormones, ScA and MJ at higher concentrations affected the production of secondary carotenoids but each with different mechanism of inhibition. ScA affected the secondary carotenogenesis mainly by scavenging the free radicals necessary for secondary carotenoid induction. While MJ increased the production of primary carotenoids especially β-carotene and lutein, thereby secondary carotenoid production is affected. At low concentrations these phytohormones could be used as elicitors for secondary carotenoid production.

Chapter V

Expression of carotenogenic genes and associated changes in pigment profile during regeneration of Haematococcus pluvialis cysts

Summary

Haematococcus pluvialis is a green alga known to accumulate astaxanthin in cyst cells under stress conditions which have the ability to regenerate under favorable conditions. The present study revealed the influence of few cultural parameters and temperature treatments on regeneration efficiency of red cysts along with changes in pigment profile and expression of carotenogenic genes during regeneration. Regeneration efficiency has been improved by incubating less aged cyst cells in medium containing ammonium carbonate, 16:8 light dark cycle with light intensity of 30 μmol m^{-2} s^{-1}. During regeneration there was decrease in total astaxanthin, total carotenoids and carotenoid to chlorophyll ratio, and increase in β-carotene, lutein, total chlorophyll and chlorophyll a to b ratio. Expression analysis revealed the presence of transcripts of carotenogenic genes, *PSY*, *PDS*, *LCY*, *BKT* and *CHY* in cyst cells and these transcripts were up-regulated transiently upon transfer to favorable conditions. As the culture growth progressed, carotenogenic genes expression were decreased and reached basal expression levels of green motile vegetative cells. In addition, this is the first report of detection of carotenogenic gene transcripts in red cysts and their differential expression during regeneration. The present study suggests the use of red cysts as an alternate inoculum for mass cultivation to combat protozoan predation.

5.1. Introduction

Green motile cells of *H. pluvialis* dominate under optimal growth conditions and their growth and multiplication are limited to few divisions followed by formation of non-motile vegetative cells (palmella). Under stress conditions, such as nutrient deprivation, high light intensity, salinity and acetate addition, both motile and non-motile vegetative cells transform into cysts (Margalith 1999; Jin et al. 2006). During the transformation, a trilaminar sheath and acetolysis resistant material is formed and thickened, coinciding with massive accumulation of astaxanthin in extra-plastidic lipid vesicles and expansion of cell volume (Montsant et al. 2001). The cyst wall is composed of an outer primary wall, a trilaminar sheath, secondary wall and a tertiary wall. After maturation, transfer of cysts to non-stressed conditions, released many flagellated cells by germination (Triki et al. 1997; Hagen et al. 2001) and astaxanthin in these cells are degraded slowly (Fabregas et al. 2001). The major problem in outdoor cultivation is susceptibility of vegetative cells to protozoan predation while cyst cells are resistant to predation. Therefore regeneration of cyst cells to large number of flagellated cells has been explored in the present study under different conditions. Possibility of using germinated flagellated cells as starting material for cultivation scheme has been suggested by Hagen et al. (2001).

For better understanding of astaxanthin biosynthesis, knowledge on accumulation and degradation of carotenoids and their relation with expression of carotenogenic genes are necessary. Information on carotenogenesis during stress induced accumulation of astaxanthin is well documented (Sun et al. 1998; Grunewald et al. 2000; Steinbrenner and Linden 2001; Steinbrenner and Linden 2003) whereas information on changes during cyst germination is limited to astaxanthin, chlorophyll and protein contents (Kobayashi et al. 1997a; Fabregas et al. 2003). Information regarding transcriptional changes is completely lacking. So the present study has been taken up to understand the changes occurring at pigment level and expression of carotenoid genes during germination of cysts along with influence of cultural parameters and temperature treatments on regeneration of cyst cells.

5.2. Materials and methods

5.2.1. Algal culture conditions

The green motile *H. pluvialis* cells were grown in modified BBM medium as described in section 2.2.3 and were incubated at 60 μmol m^{-2} s^{-1} with addition of 0.2% NaCl and 4.4 mM sodium acetate for 3, 5 and 7 months to induce secondary carotenoids. Encysted red cyst cells were harvested by centrifugation and these cells were taken for regeneration studies.

5.2.2. Effect of N source, light cycle, age of cyst cells, and temperature treatment on regeneration

The effect of culture parameters on regeneration was tested. For regeneration, freshly harvested red cysts were inoculated into autotrophic media to a cell density of 15 × 10^4 cells ml^{-1} and exposed to the light intensity of 30 μmol m^{-2} s^{-1} at 25±1°C, and observed for their regeneration efficiency under the influence of N source (sodium nitrate - 0.24 g l^{-1}, ammonium carbonate - 0.16 g l^{-1} and potassium nitrate - 0.41 g l^{-1} in autotrophic bold basal medium), light cycle (alternate light and dark for 18:6 h or continuous light), age of cyst cells (3, 5 and 7 months old), and temperature treatment of cyst cells (0°C and 4°C for varying periods of time). Initial cyst cell count and number of cyst cells after 3 days of incubation were counted using haemacytometer to calculate regeneration efficiency. Regeneration efficiency was calculated using the formula: (Initial cell count - Final cell count)/Initial cell count × 100.

5.2.3. Growth and pigment changes during regeneration

A time course study on changes in growth and pigment profile was carried out. Three months old cyst cells were exposed to favorable conditions. Aliquots of culture were harvested at different intervals, lyophilized and weight was estimated gravimetrically. Lyophilized cells were extracted with 90% acetone repeatedly until the pellet becomes colorless and pigment contents were estimated as described in 2.2.6.

5.2.4. Extractability of carotenoids

Extractability of carotenoids from regenerating cells under favourable conditions at different intervals was studied to evaluate the fragility of cell wall. Extractable carotenoid content was estimated by treating the lyophilized cells with 90% acetone

for 1 h without any homogenization. For each sample, extraction with 90% acetone by homogenization was served as total carotenoid and carotenoid content was calculated as per Lichtenthaler (1987). Extractability was calculated by using the formula modified from Kobayashi et al. (1997a): Extractability % = extractable carotenoids % (w/w)/ total carotenoids % (w/w) × 100.

5.2.5. RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cyst cells were exposed to favorable conditions for regeneration. At different intervals, 1×10^8 cells were harvested, frozen under liquid nitrogen and subsequently powdered using a mortar and pestle. RNA extraction, reverse transcription, and PCR were carried out as described in section 2.2.7. Transcript levels of each gene in green motile cells were taken for comparison in calculating the transcript abundance of respective genes during regeneration.

5.2.6. Experimental design and data analysis

Each experiment was repeated twice with three replications. The data were analyzed by one way analysis of variance and post-hoc mean separations were performed by Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

5.3. Results

5.3.1. Regeneration efficiency of *Haematococcus* cysts under the influence of cultural parameters and temperature treatments

The regeneration rate of encysted (aplanospore) *Haematococcus* cells varied based on nitrogen source, light dark cycle, age of cells and temperature treatment (Tables 5.1 and 5.2). Of the autotrophic media differing in N source evaluated for regeneration, regeneration efficiency was found to be more in medium with ammonium carbonate when compared to the media with sodium nitrate and potassium nitrate (Table 5.1).

Table 5.1. Influence of nitrogen source, light dark cycle and age of cyst cells on regeneration

Treatment	Conditions			% of cysts germinated*
	N source	Light dark cycle	Age of cysts (months)	
A	Ammonium carbonate	16:8 h	3	83.1[a]
B	Potassium nitrate	16:8 h	3	61.2[b]
C	Sodium nitrate	16:8 h	3	64.8[b]
D	Ammonium carbonate	16:8 h	3	83.7 [a]
E	Ammonium carbonate	Continuous light	3	65.5[b]
F	Ammonium carbonate	16:8 h	3	83.3[a]
G	Ammonium carbonate	16:8 h	5	41.4[b]
H	Ammonium carbonate	16:8 h	7	30.0[c]

** Each value represents the mean of two separate experiments each with three replicates. Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Maximum regeneration efficiency of 83% was observed in ammonium carbonate medium when compared to 60-65% in medium with nitrate as nitrogen source. Regeneration efficiency of cyst cells decreased with increase in age of cyst cells. Maximum regeneration of 83% was observed in 3 months old cyst cells. Under high light intensity (60 µmol m^{-2} s^{-1}) combined with other favorable conditions, cyst cells started to regenerate but soon they were bleached. Exposure of cyst cells to low light intensity with alternate light dark cycle favored faster and higher regeneration (84%) than continuous light. Data obtained on regeneration of encysted cells after pretreatment at 4 and 0^{o}C had shown that, the regeneration was more in cells exposed

to 0°C than to 4°C. Short intervals of freezing and thawing enhanced the regeneration efficiency (Table 5.2) while exposure of cells to 0°C for longer duration (1 h) injured the cells and affected the regeneration ability of cells. However, regeneration was not affected when the cells were exposed to 4°C for longer duration (1 h). Complete regeneration of cyst cells was observed over a period of time in all treatments except in cells exposed to 0°C for long time, where cells were bleached either partially or completely (Table 5.2).

Table 5.2. Influence of temperature treatments on regeneration

Treatment	% of cysts germinated**
Control	83.6 ^{ab}
0°C for 5 min	67.5 ^{e}
0°C for 10 min	71.1 ^{be}
0°C for 5 min followed by 10 min incubation at 30°C for 3 cycles	87.7 ^{a}
0°C for 10 min followed by 10 min incubation at 30°C for 3 cycles	44.4 ^{g}
0°C for 1 h	Cells bleached
4°C for 5 min	77.2 ^{d}
4°C for 10 min	61.8 ^{f}
4°C for 30 min	80.2 ^{bcd}
4°C for 5 min followed by 10 min incubation at 30°C for 3 cycles	81.7 ^{bc}
4°C for 1 h	79.0 ^{cd}
4°C for 5 h	57.9 ^{f}

** Each value represents the mean of two separate experiments each with three replicates. Means within a column followed by same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

5.3.2. Growth and pigment changes during regeneration

Growth of regenerated cultures were estimated as dry biomass per litre, and showed initial slight decrease and further constant increase during regeneration (Figure 5.1). In the first day itself, very few cells, which were fast moving and flagellated were observed microscopically. In the encysted cells, total carotenoid content was 1.9 to 2.0% on dry weight basis. Astaxanthin constituted 85-90% of total carotenoids of which monoester constituted 71.8%, diester 27.7% and around 0.5% free astaxanthin. During regeneration in the autotrophic medium although the astaxanthin content decreased significantly, components of astaxanthin i.e. astaxanthin monoester, diester

and free astaxanthin ratio did not show much variation. Their contents were ranged from 73.8-63.5% for monoesters, 32.8-25.9% for diesters and 3.6-0.3 for free astaxanthin. In the first few days total carotenoid content (w/w) increased marginally followed by a decrease while chlorophyll pigments showed a continuous increase (Figure 5.2). Chlorophyll a to b ratio (chl a/b) increased with a concomitant decrease in carotenoid to chlorophyll ratio (car/chl). As the cyst cells started regeneration, there was significant decrease in astaxanthin content with corresponding increase in lutein (major component) and β-carotene, and very low quantities of canthaxanthin, echinenone (intermediates in the formation of astaxanthin from β-carotene), neoxanthin and violaxanthin were also detected (Figure 5.3). As regeneration progressed, the chlorophyll and carotenoid content in the germinated cells reached to that in green motile cells. After complete regeneration also traces of astaxanthin was detected upto 2-3 sub culturing in nutrient sufficient medium.

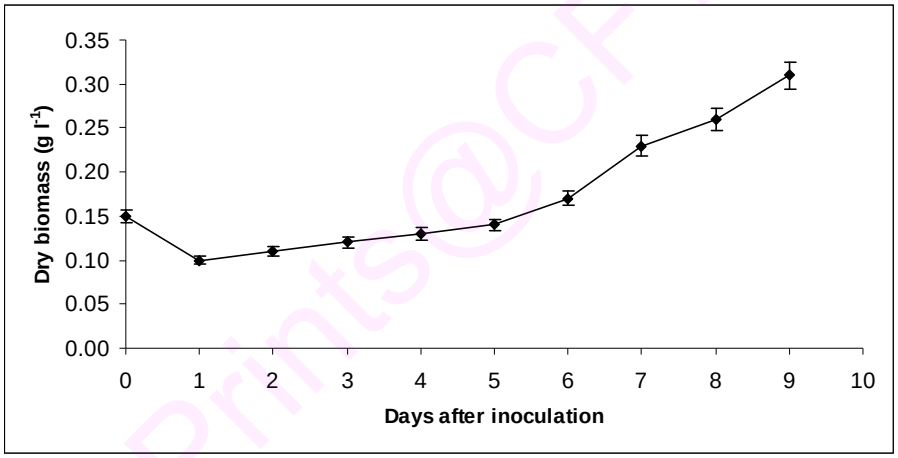


Figure 5.1. Growth changes during regeneration of *H. pluvialis* cysts. Three months old cysts were exposed to favorable conditions (autotrophic medium with ammonium carbonate as N-source incubated at 25±1°C, 30 µmol m^{-2} s^{-1} light intensity with 16:8 h light dark cycle). Regenerated cells were harvested, lyophilized and biomass expressed as g l^{-1}.

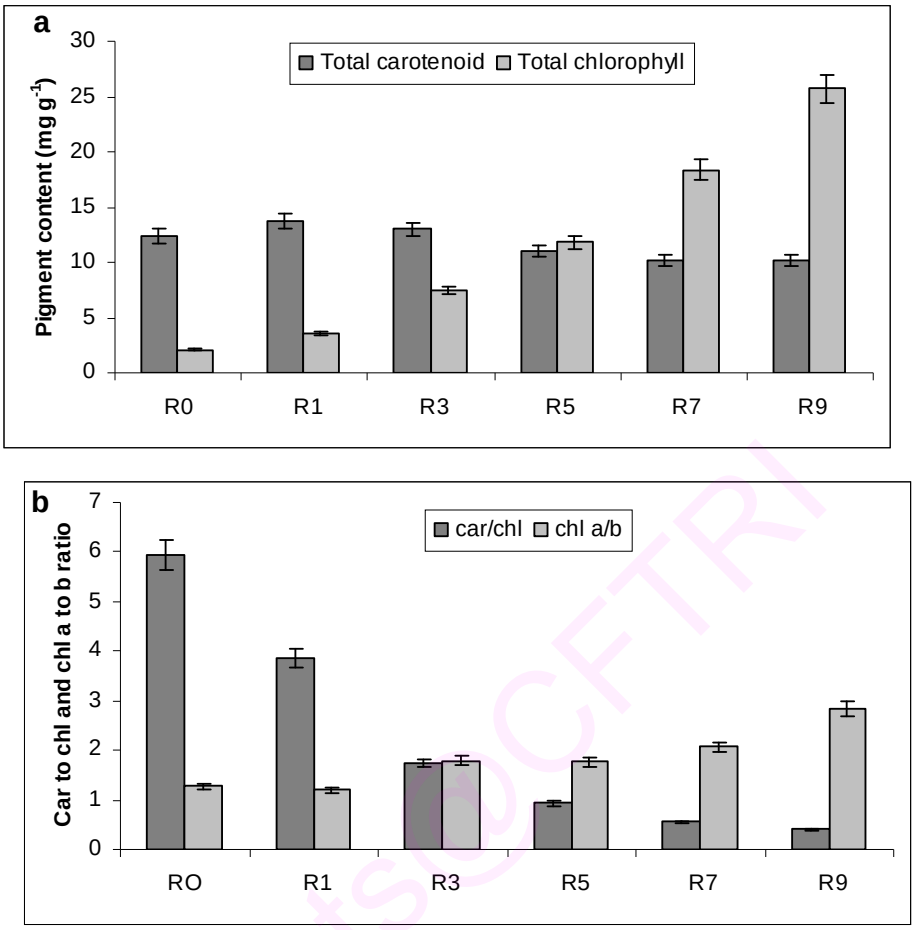


Figure 5.2. Pigment changes during regeneration of *H. pluvialis* cysts. (a) Changes in total carotenoid and total chlorophyll contents, and (b) carotenoid to chlorophyll ratio (car/chl) and chlorophyll a to b (chl a/b) ratio. Three months old cysts (R0) were exposed to favorable conditions (autotrophic medium with ammonium carbonate as N-source incubated at 25±1°C, 30 µmol m^{-2} s^{-1} light intensity with 16:8 h light dark cycle) and harvested 1d (R1), 3d (R3), 5d (R5), 7d (R7) and 9d (R9) after inoculation. Harvested cells were lyophilized and pigments were analyzed.

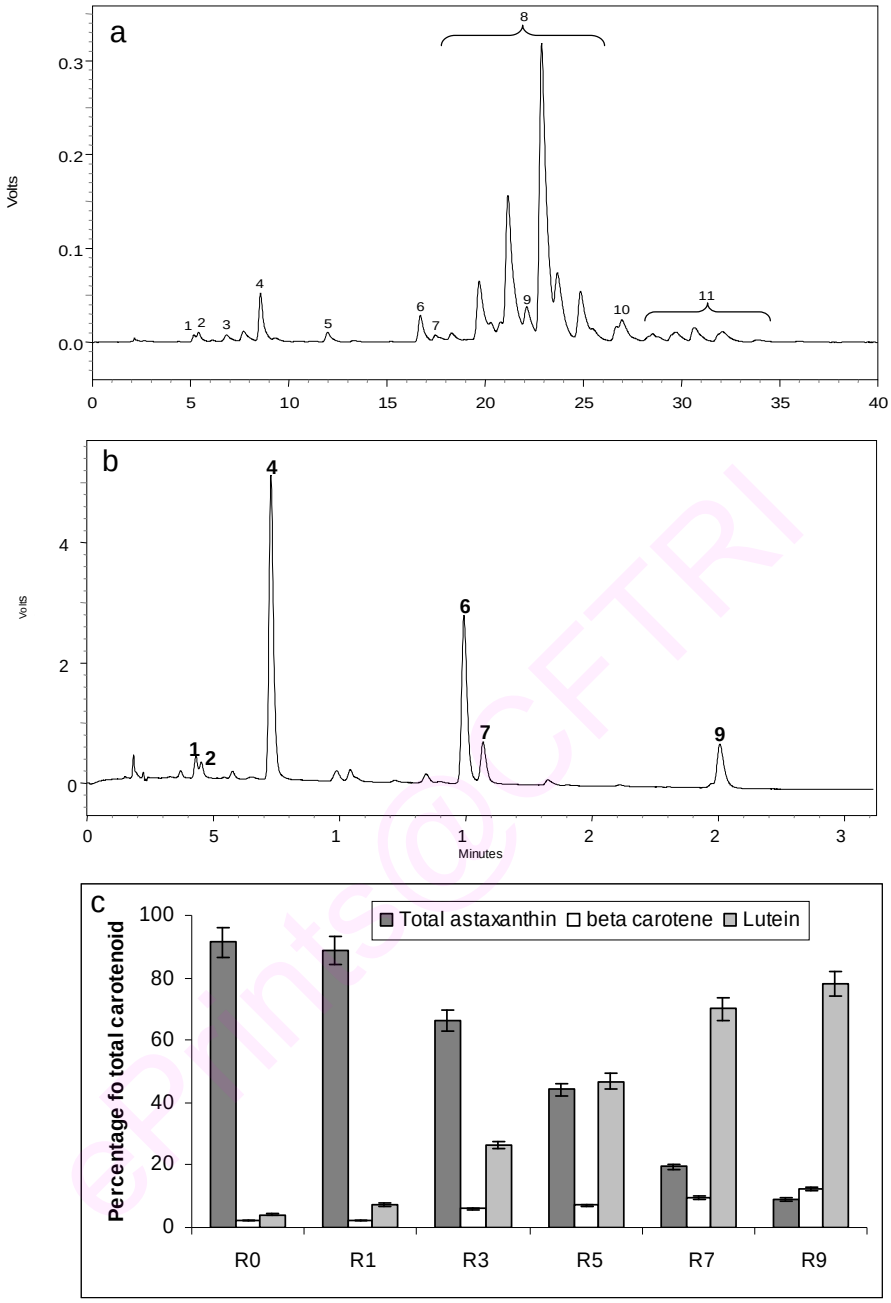


Figure 5.3. Changes in carotenoid composition during regeneration of *H. pluvialis* cysts. HPLC analysis of carotenoids extracted from (a) three months old red cyst cells and (b) regenerated green motile cells of *H. pluvialis*: 1. Neoxanthin, 2. Violaxanthin, 3. Free astaxanthin, 4. Lutein, 5. Canthaxanthin, 6. Chlorophyll b, 7. Chlorophyll b', 8. Astaxanthin monoesters, 9. Echinenone and Chlorophyll a, 10. β-Carotene and 11. Astaxanthin diesters. (c) Changes in concentration of total astaxanthin, β-carotene and lutein during regeneration of 3 months old cyst cells (R0) which were harvested 0d (R0), 1d (R1), 3d (R3), 5d (R5) 7d (R7) and 9d (R9) after inoculation.

5.3.3. Extractability of carotenoids

As shown in Figure 5.4, the extractability increased from almost nil in the cysts on the day of inoculation to 65-70% by 5-6 days of regeneration. After 6th day, extractability of carotenoids decreased to 40%. In addition, 3 days old cells had 36% extractability and 67% of the total carotenoid was astaxanthin.

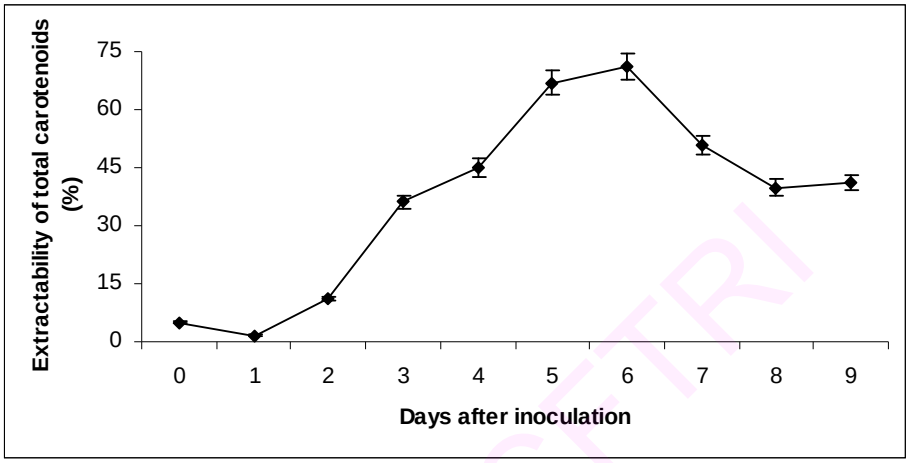


Figure 5.4. Extractability of carotenoids during regeneration of *H. pluvialis* cysts. Three months old cysts were exposed to favourable conditions. Cells at different intervals were harvested, lyophilized and extractability was estimated.

5.3.4. Changes in transcripts of carotenogenic genes during regeneration

The expression levels of genes associated with general carotenogenesis and specific astaxanthin biosynthesis during regeneration of *Haematococcus* cysts were quantified by RT-PCR and compared with the expressions of respective genes in green motile cells. These genes included *PSY*, *PDS*, *LCY*, *BKT* and *CHY*. Transcripts of *PSY*, *PDS*, *LCY*, *BKT* and *CHY* were detected in three months old red cyst cells of *Haematococcus* (Figure 5.5). In addition, this is the first report of carotenoid genes expression in red cysts and their differential regulation during regeneration.

Exposure of cysts to favorable conditions (autotrophic medium with ammonium carbonate as N-source, 25±1°C under 30 µmol m^{-2} s^{-1} light intensity with 16:8 h light dark cycle) induced *PSY* expression transiently (from 1.5-fold to 9.3-fold) on the 1st day of exposure to favorable conditions, and considerably decreased on 5th day, and remained low later on. The expression of *PDS* showed a transient increase from 1.4-fold to 4.8-fold on 1st day of regeneration, thereafter showing decreasing trend. Transcripts of *LCY* were up-regulated and reached 8.0-fold increase from 5.3-fold on 3rd day, and then decreased. *BKT* transcripts have shown 8.0-fold increase on

1st day. The expression of *CHY* was reduced immediately upon exposure to favorable conditions (from 7.8-fold to 3.2-fold compare to green motile cells). On 7th day of regeneration, expressions of all carotenoid genes studied reached basal expression levels of green motile cells.

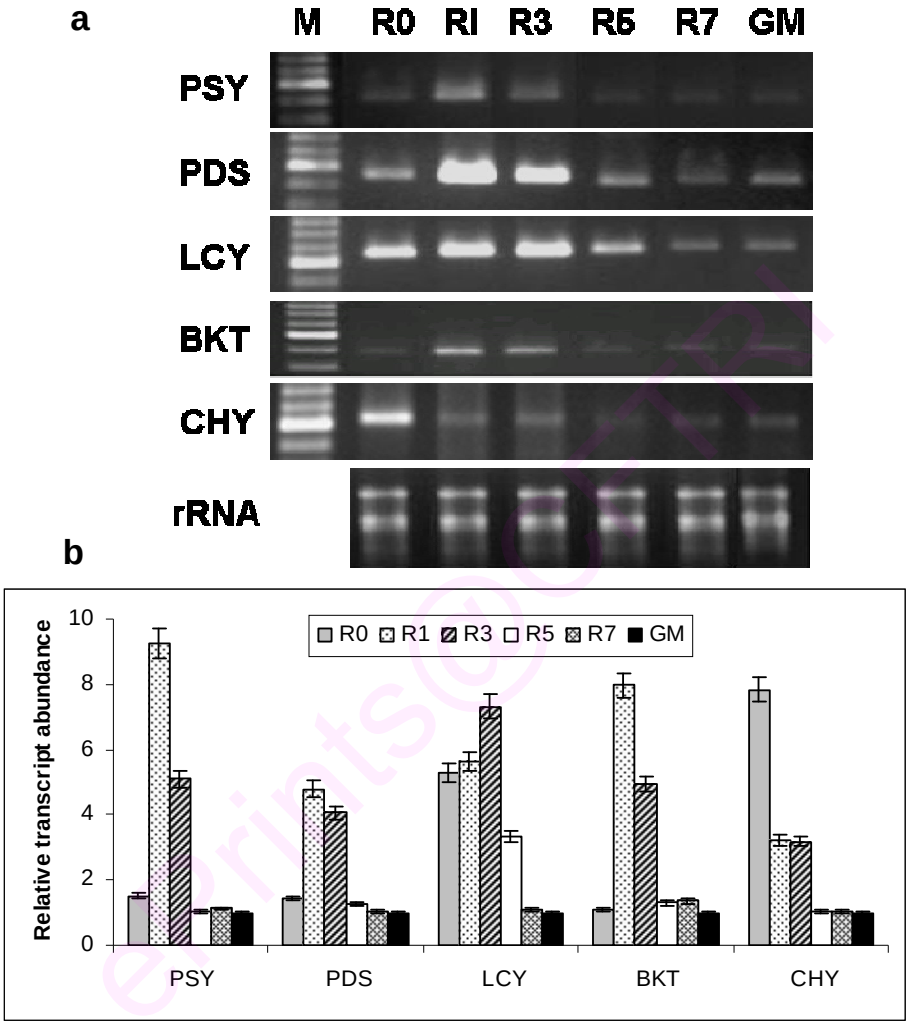


Figure 5.5. Transcripts of carotenogenic genes were up-regulated transiently during regeneration of *H. pluvialis* cysts. The regenerating *H. pluvialis* cells were harvested at 0d (R0), 1d (R1), 3d (R3), 5d (R5) and 7d (R7) after exposure to favorable conditions, and RNA was isolated. RT-PCR was performed as described in materials and methods. (a) The PCR products were analysed by agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (lower panel). M, 100 bp DNA ladder plus (Fermentas). (b) Data shown are mean ± SD of 3 independent experiments expressed as the fold increase in *PSY*, *PDS*, *LCY*, *BKT* and *CHY* expression levels compared with the value for green motile cells (GM).

5.4. Discussion

The present study was undertaken to evaluate the methods for achieving maximum regeneration rate of cysts, and to study changes in carotenogenesis during regeneration. The results showed the influence of nitrogen source, light dark cycle, age of cells and treatment temperature on regeneration efficiency of cyst cells. Bleaching of cells observed during regeneration of cyst cells exposed to 0°C for long time may be due to internal ice crystal formation in absence of added cryoprotectants thereby affecting the regenerating ability of cells. The complete regeneration obtained in cells treated at 4°C for different durations indicates that cells can withstand that temperature without losing the viability. Earlier reports had shown the germination of aplanospores in dark (Hagen et al. 2001; Fabregas et al. 2003), urea-enriched medium (Lee and Ding 1994), and N-sufficient medium, and aplanospore germination was not induced in N-free cultures (Fabregas et al. 2003). During regeneration, there was a decrease in total astaxanthin content; however the relative proportion of free, mono and diester forms of astaxanthin did not show much variation. This indicated active degradation of astaxanthin esters. Fabregas et al. (2003) reported that nutrient availability was a main factor triggering the degradation of astaxanthin, while light intensity has no effect on the loss of astaxanthin during germination.

The cyst cells contain a thick cell wall made up of algaenan which hinders carotenoid extraction by solvents. This also reduces carotenoid bioavailability when whole intact cells were used in nutraceutical preparations. Therefore the cells require homogenization under high pressure at cryogenic conditions or cracking before usage. However during regeneration, the extractability of carotenoids increased significantly. This could be due to breakage of algaenan containing trilaminar sheath, secondary wall and tertiary wall during germination as reported by Damiani et al. (2006). This characteristic feature is having biotechnological importance since pigment extraction from flagellated cells becomes easier (Hagen et al. 2001).

As the growth progresses, transcripts of carotenogenic genes studied - *PSY*, *PDS*, *LCY*, *BKT* and *CHY* were decreased and their levels reached basal expression level of green motile cells (Figure 5.5). This is in concordance with the report of Huang et al. (2006a) for basal expression of *BKT* in green flagellated cells. Even though transcripts of *BKT* were detected in green motile cells in our study, the

decrease in astaxanthin during regeneration could be due to reduced level of *BKT* transcripts which might have been below a threshold amount necessary for astaxanthin biosynthesis (Huang et al. 2006a).

During astaxanthin accumulation in *Haematococcus*, it has been reported that, high light reduced the plastoquinone pool which seems to function as redox sensor for transcriptional activation of carotenogenic genes (Steinbrenner and Linden 2003). Though red cyst cells are photosynthetically competent, they operate at photosynthetically reduced level which may be due to impaired linear electron flow from PSII to PSI (Tan et al. 1995). An increase in chlorophyll, decrease in carotenoid and transient induction of carotenogenic genes observed during regeneration indicates the possible function of plastoquinone pool as an electron crossover point between photosynthesis and carotenoid synthesis, as suggested by Kobayashi et al. (1997a).

The slight increase in total carotenoid and total astaxanthin content observed immediately after exposure of cyst cells to favourable conditions correlated with transient increase in transcript levels of carotenogenic genes. This shows the organism's adaptability to new environment. Since immediately upon exposure to favourable conditions, extractability decreases, slight increase in total carotenoid and total astaxanthin may not be due to change in fragility or permeability of cell wall, and it may be the result of transient induction of carotenogenic genes. In the present study, we observed an increase in primary carotenoids and chlorophyll, and decrease in secondary carotenoids and car/chl ratio. It is reported that induction of carotenogenic genes expression and increase in total carotenoids and secondary carotenoid occur under stress conditions (Jin et al. 2006). This suggested that induction of carotenogenic genes expression occurs when cells are exposed to new conditions (temporary increase in expression under favorable conditions and higher expression under stress conditions) otherwise they are maintained at basal expression level.

Chapter VI

Summary and Conclusions

6.1. Brief Background

Chlorophylls and carotenoids are the most abundant pigments in nature. Carotenoids provide many fruits and flowers with distinct red, orange and yellow colors and a number of carotenoid-derived aroma, and they are ubiquitous component of all photosynthetic organisms as they are required for assembly and function of the photosynthetic apparatus. In plants, carotenoids participate in the light harvesting process as accessory light-harvesting pigments and they protect the photosynthetic apparatus from photo-oxidative damage, besides acting as membrane stabilizers (Bartley and Scolnik 1995). Animals do not synthesize carotenoids de novo and rely upon the diet (Demmig-Adams and Adams 2002). In animals, besides acting as precursors for vitamin A and retinoids, carotenoids act as quenchers of singlet oxygen, free radical scavengers and antioxidants, and they show protective role against some cancers and macular degenerative diseases (Fraser and Bramley 2004; Stahl and Sies 2005).

Microalgal species are being exploited for large scale production of carotenoids. They produce wide spectrum of carotenoids like lutein, zeaxanthin, astaxanthin, canthaxanthin and β-carotene (Bhosale and Bernstein 2005). *Haematococcus pluvialis* is a richest source of valuable ketocarotenoid – astaxanthin which is accumulated as a secondary carotenoid in extra plastidic lipid vesicles under stress conditions such as high light, salt stress and acetate addition (Lorenz and Cysewski 2000; Grünewald et al. 2001). The two distinct phases in the life cycle of *H. pluvialis* - a green motile vegetative phase and a non-motile astaxanthin-accumulating cyst phase, favors the use of *H. pluvialis* as a model system to study the regulation of secondary carotenogenesis.

In the biosynthesis of carotenoids, the first committed step, the head-to-head condensation of geranylgeranyl diphosphate (GGPP) to phytoene, is mediated by the soluble enzyme phytoene synthase (PSY). The subsequent steps of the pathway leading to the synthesis of colored carotenoids are carried out by membrane-localized enzymes such as phytoene desaturase (PDS) and lycopene β-cyclase (LCY) (Cunningham and Gantt 1998). The biosynthesis of astaxanthin in *Haematococcus* follows the general carotenoid pathway up to β-carotene formation. β-carotene ketolase (BKT) converts β-carotene to canthaxanthin via echinenone which is further

acted upon by β-carotene hydroxylase (CHY) resulting in the formation of astaxanthin (Jin et al. 2006). Studies on expression of carotenogenic genes revealed that they are transcriptionally up-regulated. Although there are reports available on the influence of light on the expression of carotenogenic genes in *Haematococcus,* studies on the influences of other stress factors on the expression of carotenogenic genes are limited (Grünewald et al. 2000; Steinbrenner and Linden 2001; Huang et al. 2006a). Under nutrient-limiting conditions, the induction of astaxanthin accumulation occurs in flagellated cells (Brinda et al. 2004), which is more advantageous for biochemical analysis and for extraction of pigments as the cells are fragile compared to cyst cells. Moreover astaxanthin accumulation process can be studied independent of the processes involved in aplanospore formation. Understanding the molecular basis of stress-induced astaxanthin accumulation in *Haematococcus* will be useful for the optimization of astaxanthin production. With this background knowledge, the objectives of the present research work were laid as follows:

1. To study the differential expression of the enzymes involved in astaxanthin biosynthesis in *Haematococcus pluvialis*
2. To study the biochemical changes associated with carotenogenesis

6.2. Summary of results

6.2.1. Expression of carotenoid biosynthetic genes and carotenoid accumulation

Studies on changes in the pigment profile, expression of carotenogenic genes and their regulation in *H. pluvialis* were carried out. The result showed that nutrient stress (modified BBM containing $1/10^{th}$ of N and P) and higher light intensity (60 μmol m^{-2} s^{-1}) in combination with 17.1 mM NaCl / 4.4 mM sodium acetate (SA) enhanced total carotenoid and total astaxanthin content to 32.0 and 24.5 mg g^{-1} of dry biomass, respectively. Expression of carotenogenic genes, phytoene synthase (*PSY*), phytoene desaturase (*PDS*), lycopene β-cyclase (*LCY*), β-carotene ketolase (*BKT*) and β-carotene hydroxylase (*CHY*) were up-regulated under all the stress conditions studied. However, the extent of expression of carotenogenic genes varied with stress conditions. Nutrient stress and high light intensity induced expression of astaxanthin biosynthetic genes, *BKT* and *CHY,* transiently. Enhanced expression of these genes

was observed with SA and NaCl/SA, while expression was delayed with NaCl. Maximum transcript levels of *PSY*, *PDS*, *LCY*, *BKT*, and *CHY* were found to be 158–277, 5–9, 470–674, 28–40, and 451–673-fold higher, respectively, than green vegetative cells. The maximum content of astaxanthin recorded in cells grown in medium with SA and NaCl/SA correlated with the expression profile of the astaxanthin biosynthetic genes. Studies using various inhibitors indicated that general carotenogenesis and secondary carotenoid induction were regulated at both transcriptional and cytoplasmic translational levels. The induction of general carotenoid synthesis genes was independent of cytoplasmic protein synthesis while *BKT* gene expression was dependent on *de novo* protein synthesis. The present study also suggests a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes.

6.2.2. Regulation of carotenogenic and photosynthetic genes expression

Regulation of expression of carotenoid biosynthetic genes and photosynthetic genes were studied using carotenoid and fatty acid synthesis inhibitors and photosynthetic electron transport inhibitors. Various carotenoid synthesis inhibitors such as diphenylamine (DPA), nicotine, norflurazon (NF) and fatty acid synthesis inhibitor cerulenin were used to study the regulation of carotenogenesis under high light intensity of 97 µmol m^{-2} s^{-1} in nutrient sufficient (NS/HL) and nutrient deficient (ND/HL) media. The results showed inhibition of astaxanthin by all the inhibitors under NS/HL and ND/HL. Total carotenoid contents were reduced to 0.70, 0.53, 0.59 and 0.79-fold respectively of NS/HL control in DPA, nicotine, NF and cerulenin treated cultures while the total carotenoid contents were reduced to 0.37, 0.61 and 0.61-fold respectively of ND/HL control in nicotine, NF and cerulenin treated cultures except DPA where enhanced total carotenoid production through accumulation of β-carotene and lutein was observed. Lycopene accumulation was observed with nicotine treatment while β-carotene accumulation was observed with DPA. Expression of carotenogenic genes *PSY* was enhanced by all inhibitors studied except DPA while *PDS* expression was reduced by all inhibitors tested except nicotine and the expression of *LCY* was enhanced in all the inhibitor treatments. Reduced expressions of both *BKT* and *CHY* were observed under DPA and NF treatment while enhanced expression for *BKT* was observed under nicotine treatment and *CHY* expression under nicotine and cerulenin. Exposure of cells to ND/HL enhanced carotenogenic genes

expression when compared to respective inhibitors to NS/HL in most cases. Reduced expression of *LCY* of nicotine and *PSY* and *LCY* of cerulenin treated cultures resulted in increased lycopene accumulation and decreased β-carotene and lutein respectively. Non correlation of reduced carotenoid levels and enhanced expression of carotenoid genes at one hand and accumulation of lycopene and β-carotene which are intermediates in astaxanthin biosynthetic pathway observed under the experimental conditions on the other hand indicate the involvement of other regulatory factors besides transcriptional regulation of carotenogenesis in *H. pluvialis*.

Pigment accumulation, maximum quantum yield of photosystem II (PSII), and differential expression of photosynthetic genes were studied under stress conditions. Regulation of transcription of photosynthetic genes was also studied using inhibitors of photosynthetic electron transport, transcription, translation as well as carotenoid synthesis. Astaxanthin accumulation is accompanied by reduction in chlorophyll content, PSII quantum yield and total carotenoid, and increase in chlorophyll a/b ratio. Reduced chlorophyll a/b ratio was observed in cysts. Expression of photosynthetic genes, *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* were studied. The nuclear genes *cabL1818* and *lhcbm9* codes for the chlorophyll *a/b* binding protein L1818 and major light-harvesting complex II m9 protein respectively. The chloroplast encoded *psaB* gene codes for PSI reaction centre protein PsaB. The chloroplast genes *rbcL* and *atpB* codes for large subunit of Ribulose bisphosphate carboxylase oxygenase (Rubisco) and ATP synthase β-subunit respectively. Transcripts of *cabL1818*, *lhcbm9*, *psaB*, *rbcL* and *atpB* were up-regulated during stress-induced accumulation of astaxanthin with the maximum of 3.75, 1.66, 1.50, 11.63 and 1.26-fold increase respectively over non-stressed green motile cells. Transcripts of these genes were detected even in cyst cells with the transcript levels of *cabL1818*, *psaB* and *atpB* at reduced level. Studies using photosynthetic inhibitors, DCMU and DBMIB showed expression of *cabL1818*, *lhcbm9*, *psaB* and *rbcL* were under redox control of plastoquinone pool and *atpB* gene expression may be regulated at cytochrome *b6/f* complex. Expression of all five photosynthetic genes studied was regulated at transcriptional and cytoplasmic translational levels, and their expressions were reduced by norflurazon induced photo oxidative stress. Acetate modulates the HL induced expression of photosynthetic genes and it depends on redox state of cytochrome *b6/f* complex and cytoplasmic protein synthesis.

6.2.3. Molecular and biochemical changes associated with carotenogenesis

The genes differentially expressed under the stress conditions were analyzed using the mRNA differential display RT PCR (DD-RT PCR) technique coupled with silver-staining. From two populations of mRNA (green vegetative cells and cells exposed to high light intensity 97 μmol m^{-2} s^{-1}), using 7 anchored primers and 5 arbitrary primers; a total of 109 transcripts which were differentially expressed under stress conditions have been identified. After removal of false positives by re-amplification, 34 partial cDNAs were cloned, sequenced and analyzed *in silico*. Almost 50% of the DD cDNAs were either of unknown function or shared no apparent homology to any expressed sequences in the GenBank/EMBL databases. And about 50% of DD clones were similar to molecules related to general metabolism, photosynthesis, carotenoid synthesis, lipid synthesis, tetrapyrrole synthesis, transporter proteins, defense signaling and genetic information processing. In addition, one partial transcript which is homologue to *psaB* gene coding D1 protein of photosystem II have been identified for the first time in *H. pluvialis*. The differential expression patterns were confirmed by RNA dot blot, northern blot and semi-quantitative RT-PCR analyses. RNA analyses of 6 DD clones revealed that 4 genes were induced under high light stress and two genes exhibited reduced expression. The relationship between the expression pattern of these mRNA transcripts and the function of their respective encoding proteins in response to high light stress were studied. The results provide a contribution to better understanding of the changes in gene expression that accompanies the carotenogenic process in *Haematococcus* under stress conditions.

Changes in pigment profile with reference to carotenoid composition in different cell fractions, activities of carotenoid enzymes such as LCY and BKT, antioxidant enzymes and protein content were studied. LCY and BKT activities were found to increase under stress condition. Maximum activity of BKT was observed in lipid globules of stress induced cultures. Analysis of astaxanthin composition revealed the decrease in free astaxanthin and increase in astaxanthin mono and diesters. Exposure of cells to high light, nutrient deficiency along with NaCl and sodium acetate addition produced cells with astaxanthin composition of 0.95, 92.56 and 6.49% of free astaxanthin, astaxanthin monoesters and diesters respectively of total astaxanthin. Influence of CO_2 enrichment (2%) along with other stress conditions like high light, nutrient deficiency (modified BBM containing $1/10^{th}$ of N and P), 17.1

mM NaCl and 4.4 mM sodium acetate addition on carotenoid accumulation was studied. Under the influence of HL, exposure of cells to nutrient deficiency enhanced carotenoid accumulation which was further enhanced by exposure to CO_2 enriched environment and/or NaCl and sodium acetate addition. Changing the carbonate buffer in the lower compartment of 2-tier vessel to provide constant CO_2 environment was found to be efficient in enhancing carotenoid content but the proportion of astaxanthin among total carotenoid was reduced. A maximum of 30.61 mg/g of total carotenoid with 87% astaxanthin was achieved by exposing cells to high light, NaCl and CO_2 (twice buffer changes).

Influence of phytohormones such as salicylic acid and methyl jasmonate on pigment production and antioxidant enzymes was studied. At lower concentrations (10 μM), these phytohormones could be used for elicitation of secondary carotenoid production. Higher concentrations of salicylic acid and methyl jasmonate inhibited astaxanthin accumulation but each is having different mechanism of inhibition either by scavenging the free radicals or by increasing primary carotenoids production.

6.2.4. Expression of carotenogenic genes and associated changes in pigment profile during regeneration of *Haematococcus pluvialis* cysts

Influence of few cultural parameters and temperature treatments on regeneration efficiency of red cysts along with changes in pigment profile and expression of carotenogenic genes during regeneration were investigated. Regeneration efficiency has been improved by incubating less aged cyst cells in medium containing ammonium carbonate, 16:8 light dark cycles with light intensity of 30 μmol m^{-2} s^{-1}. During regeneration there was decrease in total astaxanthin, total carotenoids and carotenoid to chlorophyll ratio, and increase in β-carotene, lutein, total chlorophyll and chlorophyll a to b ratio. Expression analysis revealed the presence of transcripts of carotenogenic genes, *PSY*, *PDS*, *LCY*, *BKT* and *CHY* in cyst cells and these transcripts were up-regulated transiently upon transfer to favorable conditions. As the culture growth progressed, carotenogenic genes expression were decreased and reached basal expression levels of green motile vegetative cells. In addition, this is the first report of detection of carotenogenic gene transcripts in red cysts and their differential expression during regeneration. The present study suggests the use of red cysts as an alternate inoculum for mass cultivation to combat protozoan predation.

6.3. Conclusions

Detailed studies were undertaken to analyze the expression of carotenoid biosynthetic genes of *H. pluvialis* and their regulation along with molecular and biochemical changes associated with carotenogenesis. In addition, changes in pigment profile and expression of carotenoid biosynthetic genes during regeneration of *H. pluvialis* cysts were also studied. Expression of carotenoid biosynthetic genes of *H. pluvialis* was studied and up-regulation of these genes correlated with enhanced astaxanthin accumulation. Both general carotenogenesis and secondary carotenoid induction were regulated at transcriptional and cytoplasmic translational levels. This study also suggested a possible involvement of acetate in the post-transcriptional modifications of carotenoid genes. Experiments using inhibitors of carotenoid and fatty acid synthesis indicated the involvement of other regulatory factors besides transcriptional regulation of carotenogenesis in *H. pluvialis*. Studies using photosynthetic inhibitors showed expression of photosynthetic genes, *cabL1818*, *lhcbm9*, *psaB* and *rbcL* were under redox control of plastoquinone pool and *atpB* gene expression may be regulated at cytochrome *b6/f* complex. By using mRNA differential display RT PCR technique, 35 differentially expressed transcripts have been identified. A partial transcript homologue to *psaB* gene coding D1 protein of photosystem II has been identified for the first time in *H. pluvialis*. LCY and BKT activities were found to increase under stress condition. Maximum activity of BKT was observed in lipid globules of stress induced cultures. Under the influence of high light, exposure of cells to nutrient deficiency enhanced carotenoid accumulation which was further enhanced by exposure to CO_2 enriched environment and/or NaCl and sodium acetate addition. Changing the carbonate buffer in the lower compartment of 2-tier vessel to provide constant CO_2 environment was found to be efficient in enhancing carotenoid content. Expression analysis of carotenogenic genes during regeneration of *H. pluvialis* cysts showed these transcripts were transiently up-regulated upon transfer to favorable conditions and later reached basal expression levels of green motile vegetative cells. Thus the thesis work has presented in detail about the regulation of carotenogenesis - both general and astaxanthin specific under the influence of stress conditions correlating the expression of carotenogenic enzymes with metabolites. Inhibitor studies suggested regulation of carotenogenic genes at transcriptional, translational

and post-translational levels. It is evident from the results that acetate has a role in regulation of both carotenogenic and photosynthetic gene expression.

In recent years, pathway engineering of higher plants to produce valuable carotenoids is gaining importance. Therefore the results from the present studies will be helpful in understanding the regulation of carotenogenesis and metabolic engineering of carotenoid pathway in other organisms.

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Publications

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1. **Vidhyavathi R**, Venkatachalam L, Sarada R, Ravishankar GA (2008) Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. **Journal of Experimental Botany** 59 (6): 1409-1418.
2. **Vidhyavathi R,** Venkatachalam L, Kamath BS, Sarada R, Ravishankar GA (2007) Differential expression of carotenogenic genes and associated changes in pigment profile during regeneration of *Haematococcus pluvialis* cysts. **Applied Microbiology and Biotechnology** 75: 879-887.
3. Sarada R, **Vidhyavathi R**, Usha T, Ravishankar GA (2006) An efficient method for extraction of astaxanthin from green alga *Haematococcus pluvialis* **Journal of Agricultural and Food Chemistry** 54: 7585-7588.
4. Sandesh Kamath B, **Vidhyavathi R**, Sarada R, Ravishankar GA (2008) Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants. **Bioresource Technology** 99:8667–8673.

Patents

1. **Vidhyavathi R**, Sarada R, Sandesh Kamath B, Ravishankar GA. A method for production of contaminants free algal biomass-*Haematococcus*. 333/Del/2006
2. Sandesh Kamath B, Sarada R, **Vidhyavathi R**, Ravishankar GA. A process for obtaining water dispersible astaxanthin composition. 712/Del/2007

Book Chapters

1. Sarada R, **Vidhyavathi R** and Ravishankar GA (2008) Carotenoid production and characterization in cultured *Haematococcus pluvialis.* To be published in '**Protocols on Algal Research**'.

Manuscripts in pipeline

1. **Vidhyavathi R**, Sarada R, Ravishankar GA (2008) Differential expression of photosynthetic genes and their regulation during secondary carotenogenesis in *Haematococus pluvialis.*
2. **Vidhyavathi R**, Sarada R, Ravishankar GA (2008) Influence of carotenoid biosynthetic inhibitors on expression of carotenoid genes in *Haematococus pluvialis.*
3. **Vidhyavathi R**, Venkatachalam L, Sarada R, Ravishankar GA (2008) Identification and characterization of mRNA transcripts differentially expressed in

Haematococcus pluvialis during secondary carotenogenesis by differential display RT-PCR.

4. **Vidhyavathi R**, Sarada R (2008) Effect of salicylic acid and methyl jasmonate on antioxidant systems of *Haematococcus pluvialis.*

Papers presented in symposia / conferences

1. **Vidhyavathi R**, Sarada R and Ravishankar GA (2005) Nutrient stress induced accumulation of astaxanthin in *Haematococcus pluvialis*. Presented at 17th Indian Convention of Food Scientists and Technologists. NIMHANS convention centre, Bangalore. 9-10th December 2005. AP-27.
2. Sarada R, Sandesh Kamath B, **Vidhyavathi R**, Brinda BR, Ravishankar GA (2006) Astaxanthin production from green alga *Haematococcus pluvialis* under autotrophic and heterotrophic conditions. Presented at International Conference on Applied Phycology held during 14-15th February 2006.
3. **Vidhyavathi R**, Sarada R, Ravishankar GA (2007) Profile of carotenogenic genes expression and carotenoids during stress induced accumulation of astaxanthin in *Haematococcus pluvialis*. Poster presented at 8th Agricultural Science Congress, National Academy of Agricultural Sciences, STS-037 28pp, February 15th -17th held at TNAU, Coimbatore
4. **Vidhyavathi R,** Sarada R (2007) Effect of salicylic and methyl jasmonate on antioxidant systems of *Haematococcus pluvialis*. Presented at International Conference on New Horizons in Biotechnology (NHBT) held during November 26-29 in Trivandrum, India. FAB 018, 242pp
5. **Vidhyavathi R**, Sarada R, Ravishankar GA (2008) Identification and characterization of mRNA transcripts differentially expressed in response to high light by means of differential display in *Haematococcus pluvialis* Poster to be presented at a National Conference "Current Trends in Algal Biodiversity and Biotechnology" to held at CAS in Botany, University of Madras, Chennai from 7-8th February 2008

Appendix

List of the differential amplicons, accession numbers and their sequences

Fragment ID	GenBank Accn no.	Sequences (5'-3')
2.1	GE649920	CTGAGCTACCAACGGTGGCACGGGGTCGTAACGCGTATGCAATCTGCCTTAC ACCGGGGAATAGCCCAGAGAAATTTGGATTAATACCCTATAGTATACTGATG TGGCATCACATTAATATTAAAGATTTATCGGTGTAAGATGAGCATGCGTCCC ATTAGCTAGTTGGTATGGTAACGGCATACCAAGGCGATGATGGGTAGGGGTC CTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACG GGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCAT GCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTATACAGGAA GAAACCCCACTACGTGTAGCGGACTGACGGTACTGTAGGAATAAGGATCGGC TAACCCCGTGCC
H4.1	GE649921	GGCACGGGACATGCCGTTGATTTGGGAGCTCTCCCAAGCACTGCTGACAGTG ATGCGCACGGAGTTCCCCCTAAAAAAATAAAAGCCGGGAACACCCGGATGTC AGGTTCAGGGCAGGGGGCGCGGGTTGCTTTCTTCTTTTTAGTTGGTTATATA GTCGGCCTCATTGATTCATCTCTTAATTCAGCCTAAAATAAAGCAGAACACA GCATGAAACTGACGCGGAAGCACAGCAAAAGTAAGGAAGAGGTCCGGGAGGC GCTGGGGATTATTGCCGGCGATATGCAGAAAAAGTTTGGGATTGGCCACAAG TGGGACGGCGACACCCTGCGGTTTAACGGCAGCCTCGTATCCGGCTATATCG CCAATTCGGGCGATGAAATCACGGTTGATCTCAAGAAGAGTTTTTTCCTGCC CGTGCC
H7.1	GE649922	GGCACGGGCGGAGAGCCGGACTTCCCTGTTCCATCGCCGGAGAACAAGGTAC TGCCGTCGGCTTGTAGCGACGCCTTGCCGCTGTGGCTTAGTTTCTTCTTGGC TGGTTTCTTGCCGGAGTGACCGGACGCCGCCGCAGCCGCGATCTTGGCCGCC TTGTTCGCCGGGATGCCTTGCTCCTTCAACGAGCGCAACTTGCTCTTCGTGG TGCGAATGGCTTTGGAAAACTCTTCCGTCTCTTGTTGCAAAGCATCACGGCG CTGCCGCTTGGCTCCTGGTCCGCCTGCCTCGACAGCCTCGACTTTTCGTTTC TCGCGCTTCTTGTTCTTTGCTTCCTGCTTAGCCCCGTGCC
H7.2	GE649923	GGCACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCGAGCTGATGA CTCGCGCTTACTAGGCATTCCTCGTTAAAGACTAATAATTGCAATAATCTAT CCCCATCACGATGCAGTTTCAAAGATTACCCGGACCTCTCGGCCAAGGATAG GCTCGCTGAATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGC ATCACAGACCTGTTATTGCCTCACACTTCCACTGACTAAACGTCAATAGTCC CTCTAAGAAGTCAGGCGCGTACCAAGGTACGCTTGACTATTTAGCAGGCTGA GGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCCACCAACTAAGAA CGGCCATGCACCACCACCCACAGAATCAAGAAAGAGCTCTCAATCTGTCAAT CCTTCCCGTGTCTGGACCTGGTAAGTTTTCCCGTGTTGAGTCAAATTAAGCC GCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCC TTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCATAAGGTGC TGGCAGGGTCATCAATAAAACGCCTGCCAATCCCTAGTCGGCATCGTTTATG GTTGAGACTACGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTT GATTAATGAAAACATCCTTGGCAAATGCTTT
H8.1	GE649924	GGCACGGGCACCGACACACTAGCAGTCAAGCGACAGGGAATATGTCTCCGTC CCCGCCGGGCACAATTCCCTGTATCGGCCGGTTCTTCGCGCATTACTGCAGC GACACAGCAACACAGGAGAAAGCTTCGCAGGGACAGCTGGCGCTTTCCTGGT TCGTACAACTGGGTGCACGAGGGATAGAGGCATAGAGGACATTATGACAGTG TGCCATGCGGCCCCACCTAGCAGCTGCAGCGCTGTGTAACCAATCTACAGAT CCGTAGGCACTCCCAGCTTCATTCGAAGTTGACAAGACATGGCTGAACTACA ACATGGTGTTCACTGCACTGTGCTGCTCACGGCACACAAGTGGGCGCACGCA ACCGCGTTGCCTTACATCTACTGCATAGGCAGGTTTCGGCTCCACTGCGTGG CTGCTGCCCCGTCACCATGCTGCCGAAGCTGAGCTGCCCACGCAGCATGCTT CTCATCCACACAATAGCTGCCGCGGCACCTAATGCACCCCCCGTGCC
H9.1	GE649925	GGCACGGGTGCATACCTTGTTGAAGTTGGGTGTGGCAAGACCACCAAGGACC ACGACCGCCTTCTTTTGACTGTCATTAAGGCTGCTGATGGCTCATACCCTAC TGTTGCAGAGGTGCAAGCAGTCCACGGCGCTGCCAAGGCAGCTGACCAGCTT GGCACGGGTGCATACCTTGTTGAAGTTGGGTGTGGCAAGACCACCAAGGACC ACGACCGCCTTCTTTTGACTGTCATTAAGGCTGACTGATGGCTCATACCCTA CTGTTGCAGAGGTGCAAGCAGTCCACGGCGCTGCCAAGGCAGCTGACCAGCA

		GTTGGCCAAGGCCCGTGCC
H13.1	GE649926	GGCACGGGGCAGGTAGTGACAATAAATAACAATACCGGGCATCAATGTCTGG TAATTGGAATGAGAACAATTTAAATCCCTTAACGAGTATCCATTGGAGGGCA AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAG TTGTTGCAGTTAAAAAGCCCGTGCC
H13.2	GE649927	GGCACGGGTAGGTTCATCAAGGATCAACACTTTGGGGCGTGTCATCAGACCG CGGGCAATCGCCACTTTTTGCTGATTGCCACCGGAAAGCAGACCAATTGCCT GTTCCATCGACGGAGTTTTCACATTAAACGGACGAATGAAATCACTCACAGC CTGCTGTTCATCGGCATGCTTCAAACTGCCGCCAGCGCGGCTGAAGTAGCGC AGCGCTGTCAGCGACATGTTCTCTTTTACTGACATGCCCAACACTAAACCGT CACGTTTACGGTCTTCGGAGATATACACAATGCCGTTTGCCAGGCCATCCTG CGGTGAACGGGTAACGACTTCATGCCCATCCAGGGTGACGTAACCGCTGGTG CGCGGTAGTGCGCCGTAGAGCACTTTCATCAGTTCGGTACGACCTGCGCCCA TCAAACCAGAGACGCCCGTGCC
H22.1	GE649928	ATGGTGGAGCACGACACTCTTTAAGCGAATTGCTCCTTTAACAACTGTCATC CGAAGATGCCGCTGCATTATGCGGTATTAGCTTAAGTTTCCCTAAGTTATTC CCCACTCAAAGGTAGATTACCTACGTGTTACTCACCCGTGCC
H22.2	GE649929	TGGTGGAGCACGACACTCTGGCCAAGGATAGGCTCGCTGAATGCATCAGTGT AGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTATTGCCT CACACTTCCACTGACTAAACGTCAATAGTCCCTCTAAGAAGTCAGGCGCGTA CCAAGGTACGCTTGACTATTTAGCAGGCTGAGGTCTCGTTCGTTACCGGAAT CAACCTGACAAGGCAACCCACCAACTAAGAACGGCCATGCACCACCACCCAC AGAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTCCCGTGCC
H25.1	GE649930	GGCATTCCTCGTTAAGACTAATAATTGCAATAATCTATCCCCATCACGATGC AGTTTCAAAGATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATGGATA CTCGTTAAGGGATTTAAATTGTTCTCATTCCAATTACCAGACATTGATGCC
H25.2	GE649931	TGTCAAAGAGGCCAGCAGTGTTCAAAACGCGAGCTGGTGACTCGCGCTTACT AGGCATTCCTCGTTAAAGACTAATAATTGCAATAATCTATCCCCATCACGAT GCAGTTTCAAAGATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATGGA TACTCGTTAAGGGATTTAAATTGTTCTCATTCCAATTACCAGACATTGATGC CCGGTATTGTTATTTATTGTCACTACCTCCCCGTGCC
H31.1	GE649932	GGCACGGGTACGTAACGCGTAGGCAATCTGCCTACTCCTGGGGGATAGCCCG CCGAAAGGTGGACTAATACCGCATAACACTGCGACGTGGCACCACGATGTAG TTAAAGAATTTCGGGAGTAGATGAGCCTGCGTGTCATTAGCTAGTTGGTGCG GTAACGGCGCACCAAGGCGACGATGGCTAGGGGAGCTGAGAGGCCGATCCCC CACACTGGCACTGAGATACGGGCCAGACTCCTACGGGAGGCAGCAGTAGGGA ATATTGGGCAATGGGCGCGAGCCTGACCCAGCCATGCCGCGTGACGGATGAA GGCCTTCTGGGTTGTAAACGTCTTTTGATCGGGAAGAAAAAACTCTTGCGAG AGGAATCGACGGTACCGACTGAATAAGCACCGGCTAACCCCGTGCC
H31.2	GE649933	GGCACGGGCTTTTAACTGCAACAACTTAAATATACGCTATTGGAGCTGGAAT TACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATGGATACTCGTTAAGGGA TTTAAATTGTTCTCATTCCAATTACCAGACATTGATGCCCGGTATTGTTATT TATTGTCACTACCTCCCCGTGCC
H32.1	GE649934	GGCACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCGAGCTGATGA CTCGCGCTTACTAGGCATTCCTCGTTAAAGACTAATAATTGCAATAATCTAT CCCCATCACGATGCAGTTTCAAAGATTACCCGGACCTCTCGGCCAAGGATAG GCTCGCTGAATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGC ATCACAGACCTGTTATTGCCTCACACTTCCACTGACTAAACGTCAATAGTCC CTCTAAGAAGTCAGGCGCGTACCAAGGTACGCTTGACTATTTAGCAGGCTGA GGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCCACCAACTAAGAA CGGCCATGCACCACCACCCACAGAATCAAGAAAGAGCTCTCAATCTGTCAAT CCTTCCCGTGCC
H32.2	GE649935	GGCACGGGGAGGTAGTGACAATAAATAACAATACCGGGCATCAATGTCTGGT AGTTGGAATGAGAACAATTTAAATCCCTTAACGAGTATCCATTGGAGGGCAA GTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGT TGTTGCAGTTAAAAAGCCCGTGCC
H32.3	GE649936	GGCACGGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGGT GGTGCATGGCCGTTCTTAGTAGGTGGGTTGCCTTGTCAGGTTGATTCCGGTA ACGAACGAGACCTCAGCCTGCTAAATAGTCAAGCGTACCTTGGTACGCGCCT

		GACTTCTTAGAGGGACTATTGACGTTTAGTCAGTGGAAGTGTGAGGCAATAA CAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATG CATTCAGCGAGCCTATCCTTGGCCGAGAGGTCCGGGTAATCTTTGAAACTGC ATCGTGATGGGGATAGATTATTGCAATTATTAGTCTTTAACGAGGAATGCCT AGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACAC ACCGCCCGTCGCTCCTACCGATTGGGTGTGCTGGTGAAGTGTTCGGATTGAC TTCAGCGGTGGGCAACCTCTGCTGTCGTTGAGAAGATCATTAAACCCTCCCA CCTAGAGAAAGGAGTAGTCGTAACAATGATCCCGTGCC
H34.1	GE649937	GGCACGGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGGT GGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGGTTGATTCCGGTA ACGAACGAGACCTCAGCCTGCTAAATAGTCAAGCGTACCTTGGTACGCGCCT GACTTCTTAGAGGGACTATTGACGTTTAGTCAGTGGAAGTGTGAGGCAATAA CAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATG CATTCAGCGAGCCTATCCTTGGCCGAGAGGTCCGGGTAATCTTTGAAACTGC ATCGTGATGGGGATGGATTATTGCAATTATTAGTCTTTAACGAGGAATGCCT AGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACAC ACCGCCCCGCCCCGTGCC
H52.2	GE649938	TGGACGTCCACCACAGGTTGGCTCGGGGCCNAAGCAATAAACCTCATGTGCT GCTGAAGGTGGAACCTCCCAGCTTGCCTGTGCTGAACCACCTGTCCGTGTCC GTGCTGTGTTGAGCGTGCCTGTGTTGGTGGAACCAGCAAGCTGAGCTGAGCA AGCTGACCAGGAGCCAGGTACAGGAACCAAACCACTGTACCTACTACACGTC TGCCATGCATGTACGTGGTGCCGATGATGTGTGTATGGTCGTGATTAGCCAC ACGGGTTTGGATGGTTCGTTGGCATGAACAATGCACTGTATAGCTTATCACT GGTTGAGTGTAAAACTGATACGGTAAAAAAAAAAAAGCTTA
H53.1	GE649939	TTTGGCACGGGCACACACACCAGAAGGCTGAGCTGAAGTGGGCAATAGGCTC ACTGTCGGTAGCCTTTACAATGATGAGCTTGCAGCCAGGTTCGATTCCAACT CATGATTTATTTTGGTGTAGCATGAGGGCTGTGGGGTTCAGGAGTTGGTTCC GGATGTTTTTGTGGTGGGTTTGAAAGTGAGCGTTGGTAAAAAAAAAAAGCTT
H55.1	GE649940	CCCCGCGTAGGCTGAAAGACGTGGGCACGGGCCTTGGCCAAATGCTGGTCAG CTGCCTTGGCAGCGCCGTGGACTGCTTGCACCTCTGCAACAGTAGGGTATGA GCCATCAGCAGCCTTAATGACGGTCAAAAGAAGGCGGTCGTGGTCCTTGGTG GTCTTGCCACACCCAACTTCAACAAGGTATGCACCCGTGCC
H64.1	GE649941	GGCACGGGCACACACACCAGAAGGCTGAGCTGAAGTGGGCAATAGGCTCACT GTCGGTAGCCTTTACAATGATGAGCTTGCAGCCAGGTTCGATTCCAACTCAT GATTTATTTTGGTGTAGCATGAGGGCTGTGGGGTTCAGGAGTTGGTTCCGGA TGTTTTTGTGGTGGGTTTGAAAGTGAGCGTTGGTAAAAAAAAAAAGCTT
H69.1	GE649942	TGTACATATCAGTTGGCAAGGGTTGTAGTGACACACCGATGCGGCACCAGTG TGCTGCTGGGCGCTAACTGCAAACCAGCTGTCACTGGCACCAAGAACGACTC TGCTCCCATCAAGCTCTGTCCTGTCCACTGTGTGTCAGGCTGATGAGCTGAC CCAGCTCAAGGCTGCTTCCAGTCCAAATACACCACGGG
H69.2	GE649943	AGCTGGGTCAGCACATCAGCCTGACACACAGTGGACAGGACAGAGCTTGATG GGAGCAGAGTCGTTCTTGGTGCCAGTGACAGCTGGTTTGCAGTTAGCGCTCA GCAGCACACTGGTGCCGCATCGGTGTGTCACTACAACCCTTGCCAACTGATA TGTACATGGTACCACAGTTTTGTGCCTGAAGGGCCAAATTCTGAAATGGAAC TGATTTTCTTTCCTGAACTCGTCCAGGGCAAG
H77.1	GE649944	GGAATCAATGCCCGCCAATGGATGGCGCTGAAGCGCGTGACCTATACTCAGC CATGGAAGCAAGTGCGAGGTTTCCATGAGTAGGAGGGCGTGGGTGTCGTTGC GCAGCCTGAGGCGTGAGCCTGGGTGAAACGGCACCTAGTGCAGATCTTGGTG GTAGTAGCAAATATTCAAATGAGAACTTTGAAGACTGAAGTGGAGAAAGGTT CCATGTGAACAACGATTGGACATGGGTTAGTCGATCCTAAGAGTGTCGTGCT CCACCAT
H77.2	GE649945	ATGGTGGAGCACGACACTCTGCAAAGCCGCCCCTGCTGTAGCTAACGCGTTA AGTTTCCCGCCTGGGGAGTACAATCGCAAGAGTGAGACTCAAAGGAATTGAC AGGCGGTATTCAAGCACG
H80.1	GE649946	GGTCGGTGCCTGTGTGTAGTGAGTGGATACGTTTTAGGGTGTGTAGTAAGTA GAAACGGCGTGTGCTGAGAATCCAGCAAGAGTGTCGTGCTCCACCAT
H80.2	GE649947	TATAGAAAGTTTTTTGAAAAATATGGATGGTCTTTAAAATAACGGGTTTAAA NTAAAAAAAATAAAAGGAATTTGGGGTTCTTCTGACCATCTTTGGTGAAGAC AGTATTCCTNTTGGGAAAAAAGAGAGTATGGATTGATTTGTGAAGTAGGGTA

		AAAAGACACGAT
H88.3	GE649948	TTGGACGTGGCAAGCCCCTAGGTAGATTGGGCTAGCTCATCAGCCTGACACA CAGTGGACAGGACGGAGCTTGATGGGAGCAGAGTCGTTCTTGGTGCTATTGA CAGCTGGTTTGCAGTTAGCGCCCAGCAGCACACTGGTGCCGCATCGGTGTGT CAGTACAACCCTTGCCAACTGATATGTACATGTTACCACAGTTTTGTGCCTG ATGGGCCAAATTCTGAAATGGAGCTGATTTTCTTTCCTGAACACATTCTTTT GCAGGGCAAGCACAGTGCCCATGTTCCAGCCTTTCTTGCATCTGTTTGTGCA AGATGTAAGTGTTTTTAATCTGTAAGATGGATGGGGTTAAAAAAAAAAAGC
H97.1	GE649949	CATCGAGGTCGACCACACGTGTGGTCGATCCAAGGACGCATACCTAAACGGT ATGATAATTCCCATTCACGGCCCATATAACAGCATACACCTAATAAAAAGTG TAATACGATTAATTAGTAAAGACCACCGTTGTACAACCACTCGTCTAAAGAA GCGGCTTCCCAGATAGGATAGAAATGTAGGCCAATGGCGTTAGATGTTGGAA TAACGGCGCCTGTGATGATGTTGTTACCGTATAATAAAGAGCCTGAAACTGG TTCACGGATACCATCAATGTCCACTGGAGGAGCAGCGATGAAAGCGATAATG AATACAGAAGTTGCAGTTAATAAAGTTGGGATCGCAACA
H97.2	GE649950	CCTGAGGCTGACAGACGGTGTCTGACTCCTAGTAGAGAGCCTGTGAGGGCGG GAGAACGAGACAGGACCCGGGGTCGGATAGAGTTCGGAGCCAAGGTAACTGT CTCTGCCCTCATAGAGCCTTAAGCACCGTAAACCATACACATTCTTAGTCAG CCAGGTGTTCGAGGAAACGGTA
H97.3	GE649951	GGCACGGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTGTGGGTGGT GGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGGTTGATTCCGGTA ACGAACGAGACCTCAGCCTGCTAAATAGTCAAGCGTACCTTGGTACGCGCCT GACTTCTTAGAGGGACTACTGACGTTTAGTCAGTGGAGGTGTGAGGCAATAA CAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCACGCGCGCTACACTGATG CATTCAGCGAGCCTATCCTTGGCCGAGAGGTCCGGGTAATCTTTGAAACTGC ATCGTGATGGGGATAGATTATTGCAATTATTAGTCTTTAACGAGGAATGCCT AGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACAC ACCGCCCGTGCC
H107.1	GE649952	CTACTTCTAGTACATCCACTTTCGTGATGTGACGGGCGGTGTGTAAAGACCC GGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAACT TCATGAAGTCGAGTTGCAGACTTCAATCCGAACTGAGAACGGCTTTTTCCGA TTAGCTCCCCCTTACGGGATCGCAA
H109.1	GE649953	AGCTTGCCCTGGACGAGTTCACCCCAATCATGAATTAGTAACTTGCCTCCCG AAGGTTAGCCCAGCTACTTCTAGTACAATCCACTTTCGTGATGTGACGGGCG GTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA CTAGCGATTCCAACTTCATGAAGTCGAGTTGCAGACTTCAATCCGAACTGAG AACGGCTTTTTCCGATTAGCTCCCCCTTACGGGATCGCAACA