**UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO**

DIPARTIMENTO DI SCIENZE MMFFNN E AGRARIA

CORSO DI DOTTORATO DI RICERCA
IN BIOTECNOLOGIE VEGETALI - XXII Ciclo
BIO/04

# IMPROVING THE NUTRIENT COMPOSITION OF *CHLAMYDOMONAS REINHARDTII* TO ENHANCE THE PRODUCTION OF PHOTOSYNTHETIC ACTIVE METABOLITES: A MOLECULAR STUDY ON THE PHOTOSYSTEM II COMPLEX.

**Coordinatore:** Prof. Stefania Masci

**Tutor:** Dott. Giuseppina Rea

**Dottorando:** Antonacci Amina

*To my family*

# CONTENT

1. INTRODUCTION ..... 1
1.1. The oxygenic photosynthesis ..... 1
1.1.2. Main features of the structure and function of PSII ..... 1
1.1.3 Photosynthetic pigments. Chlorophylls and carotenoids ..... 3
1.1.4 Carotenoids and plastoquinone biosynthesis ..... 5
1.1.5 The PSII D1 core protein ..... 9
1.1.6 Effect of stressful light and temperature conditions on thylakoid structure ..... 11
1.1.7 Adaptation response of photosynthetic apparatus to high light condition ..... 13
1.2 Correlation between human health and photosynthetic compounds ..... 15
1.2.1 Applications of plant-derived phytochemicals ..... 17
1.2.2 Carotenoid benefits for human health ..... 19
1.2.3 Enrichment of nutraceutical contents in foodstuff ..... 22
1.3 *Chlamydomonas reinhardti*i as biological farm ..... 24
1.4 *Chlamydomonas reinhardtii* mutants ..... 27
1.4.1 *C. reinhardtii* NPQ mutants ..... 27
1.4.2 *C. reinhardtii* D1 mutants ..... 30
2. PROJECT AIM ..... 31
3 MATERIAL AND METHODS ..... 32
3.1 Growth conditions and cell count ..... 32
3.2 *Chlamydomonas reinhardtii* strains ..... 32
3.2.1 D1 strains ..... 32
3.2.2 NPQ strains ..... 33
3.3 Antenna size determination ..... 33
3.4 Fluorescence measurements ..... 34
3.4.1 Chlorophyll fluorescence ..... 34
3.4.2 Oxygen evolution analyses ..... 34
3.5 Quantitative and qualitative analyses of photosynthetic pigments ..... 35
3.5.1 Pigment standards and HPLC system ..... 35
3.5.2 Carotenoids and chlorophylls extraction ..... 35
3.5.3 Pigments analyses ..... 36
3.6 Chlamydomonas nucleic acids purification ..... 36
3.6.1 Genomic DNA purification ..... 36
3.6.2 RNA extraction and cDNA synthesis ..... 36
3.7 Chlamydomonas nucleic acids analyses ..... 37

3.7.1 DNA electrophoresis on agarose gel ..... 37
3.7.2 RNA electrophoresis on agarose gel in denaturant conditions ..... 38
3.8 Polymerase chain reaction (PCR) ..... 38
3.8.1 Oligonucleotides design ..... 38
3.8.2 PCR standard conditions ..... 38
3.8.3 RNA-Retrotranscription (RT) and Real-Time PCR (RT-PCR) ..... 39
3.9 Elicitation experiments: high light and high temperature treatments ..... 40
4. RESULTS AND DISCUSSION ..... 41
4.1 Selection of the strains and experimental set up ..... 41
4.2 Characterization of *C. reinhardtii* strains under physiological conditions ..... 45
4.2.1 Growth curve ..... 45
4.2.2 Chlorophyll fluorescence ..... 47
4.2.3 Oxygen evolution capacity ..... 50
4.2.4 PSII antenna size ..... 52
4.2.5 Accumulation of photosynthetic antioxidant pigments ..... 54
4.3 Elicitation strategies to improve antioxidant photosynthetic pigment accumulation ..... 56
4.3.1 Superimposition of high light and high temperature on D1 strains ..... 56
4.3.2 HL/HT effects on the chlorophyll content and PSII photochemistry in D1 strains ..... 58
4.3.3 Antioxidant photosynthetic pigment accumulation during HL/HT treatment on D1 strains ..... 59
4.4 Comparative analyses of time-course gene expression profiles in chlamydomonas mutants by Real-Time RT PCR in response to high light and high temperature treatments ..... 61
4.4.1 Expression analyses of *psb*A and *psb*D genes in D1 mutants in response to high light and high temperature treatments ..... 63
4.4.2 Expression analyses of genes involved in carotenogenisis in D1 mutant strains in response to high light and high temperature treatments ..... 65
4.4.3 Expression analyses of genes involved in plastoquinone biosynthetic pathway in D1 mutant strains in response to high light and high temperature treatments ..... 69
4.5 *C. reinhardtii* as biological farm ..... 71
5. CONCLUSIONS ..... 75
6. REFERENCES ..... 78

# 1. INTRODUCTION

## 1.1. The oxygenic photosynthesis

The oxygenic photosynthesis is the most important chemical process in biology by which plants, algae, and some bacteria and protistans, harvest light and convert it into readily utilizable energy (Kargul and Barber, 2008). In oxygenic photosynthesis, water is used as electron donor to reduce $CO_2$ to carbohydrates, generating molecular oxygen as a secondary product of the reaction. The whole process can be separated in a light dependent and a light independent reactions. Light reactions are triggered by the absorption of sunlight by photosynthetic pigments (chlorophylls and carotenoids) and are catalyzed by two separate macromolecular complexes: photosystem II (PSII) and photosystem I (PSI). A light-induced electron flow generates reducing equivalents ($NADPH_2$) and the proton motive force that drives the production of ATP (Mitchell, 1961). Oxygen is released by the oxygen evolving complex (OEC) that is constituted by proteins and a cluster of manganese ions. It is located on the lumenal side of the thylakoid membrane and it is associated to both the reaction centre and the cytochrome-$b_6f$ complex, a dimeric integral membrane protein complex that mediates electron transport between PSII and PSI (Figure 1).

In photosynthetic eukaryotes, both light and dark reactions take place in a specialised organelle called chloroplast.

### 1.1.2. Main features of the structure and function of PSII

PSII is a multisubunit chlorophyll–protein complex that drives electron transfer from water to plastoquinone using energy derived from light. PSII is embedded into the thylakoid membranes as well as all the complexes that catalyze the light reactions. Its native form is surrounded by the light-harvesting complex (LHCII complex), consisting of more than 30 proteins and thus called the PSII–LHCII super complex (Figure 1).

At the centre of this complex is the reaction centre (RC), which is composed of the D1 and D2 heterodimer (encoded by the chloroplast *psb*A and *psb*D genes, respectively). This heterodimer binds cooperatively to the primary chlorophyll donor P680 and to several small polypeptides (Lucinski and Jackowsky, 2006). D1 and D2 bind all cofactors involved in PSII mediated electron transport: Tyr161 ($Y_Z$), pheophytin a (Phe), a plastoquinone tightly bound to the binding pocket on D2 ($Q_A$), a plastoquinone loosely bound to D1 ($Q_B$), the Mn cluster $[Mn]_4$ of OEC, and the non-haem iron, along with two peripheral chlorophylls a and one β-carotene (Minagawa and Takahashi, 2004).

Two core antenna proteins, CP43 and CP47 surround the RC as several additional small polypeptides. In addition, three and two all trans-β-carotenes are assigned on CP43 and CP47, respectively, and there might be more carotenes on the core antenna complexes (Barber, 2006). On the lumenal side of PSII, at least three extrinsic polypeptides, PsbO, PsbP, and PsbQ, as well as several other low molecular weight subunits function as an oxygen evolving enhancer in chlorophytes (Minagawa and Takahashi, 2004). Moreover, six individual proteins (Lhcb1-6) binding an array of chlorophyll a, chlorophyll b, lutein, violaxanthin, zeaxanthin and antheraxanthin molecules form the peripheral LHCII complexes (Lucinski and Jackowsky, 2006).

The electron flow through PSII begins with the release of an electron from an excited P680 molecule (Figure 1). When the primary donor P680 is excited by light energy captured by antenna pigments, the primary charge separation takes place between P680 and the intermediate acceptor Phe; this reaction generates the radical pair ($P680^+/Phe^-$). Reduced Phe transfers an electron to the primary acceptor $Q_A$ to generate $Q_A^-$, and subsequently reduces the secondary acceptor $Q_B$. The non-heme iron ($Fe^{2+}$) laying between $Q_A$ and $Q_B$ is essential for mediating the electron transfer from $Q_A$ to $Q_B$, but is not directly involved in the redox reaction (Lucinski and Jackowsky, 2006).

The second photochemical reaction coupled with two stromal protonations generates doubly reduced $Q_B$, $Q_BH_2$ (a plastoquinol), which is released from the binding pocket and diffuses freely in the lipid bilayer of thylakoid membrane toward the cytochrome $b_6f$ complex. On the donor side, P680+ is reduced by the immediate electron donor, $Y_Z$, and the resulting neutral radical $Y_Z^\bullet$ is reduced by an electron from a cluster of four manganese atoms (Mn-cluster) involved in oxygen evolution. A mutation of Tyr161 on D1 abolishes oxygen-evolving activity in chlamydomonas (Minagawa et al., 1996). The manganese cluster accumulates four oxidizing equivalents to split two water molecules into one oxygen molecule and four protons. This linear electron transfer reaction in PSII catalyzes the light-induced water–plastoquinone oxidation–reduction with a high quantum yield. In addition to the main linear electron transfer, it has been proposed that there is a low quantum yield cyclic electron transfer around PSII, which may protect PSII against photoinhibition by preventing over-reduction of $Q_A$ and $Q_B$ on the acceptor side and accumulation of long-lived $P680^+$ on the donor side (Diner and Rappaport, 2002).

According to the Q-cycle theory (Hope, 2000; Crofts 2004; Allen 2004; Osyczka et al., 2005), only one of the two electrons that $PQH_2$ can donate, goes to the FeS protein, whereas the other one is transferred to the high and then to the low potential cyt $b_6$ heme (Figure 1). After re-

oxidation of a second $PQH_2$ molecule, both hemes become reduced and a PQ molecule on the stromal side of cyt $b_6f$ is doubly reduced. The $PQ^{2-}$-molecule picks up two protons from the stromal side and diffuses to the lumen side of the cyt $b_6f$ complex. In this way the Q-cycle increases the number of protons released into the lumen per transferred electrons.

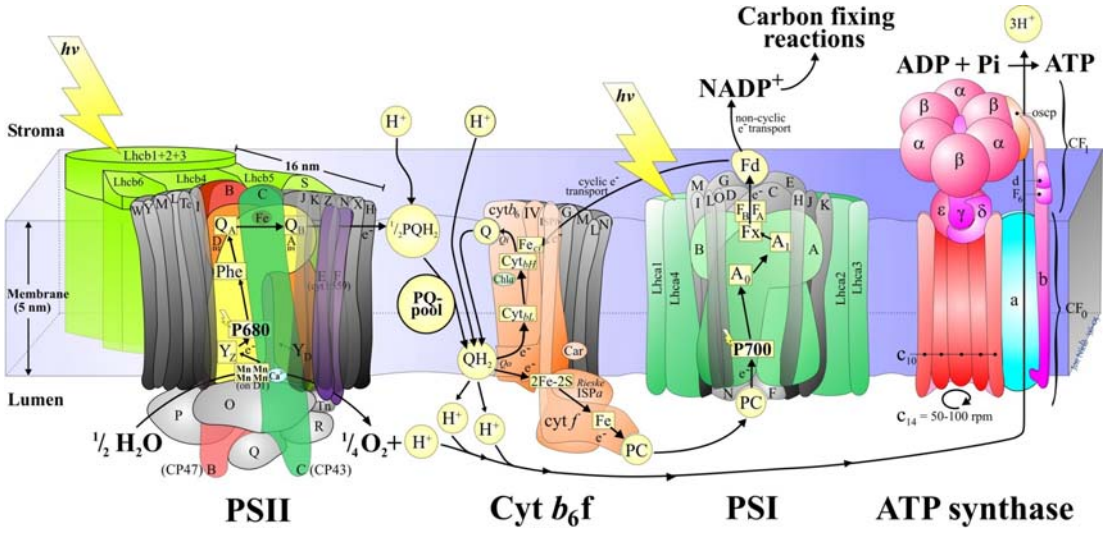



**Figure 1. Schematic model of photosynthetic electron transfer chain including structural information on the organization of the protein complex involved.** (Adapted from Jon Nield, Mechanistic and Structural Biology, SBCS, Queen Mary, University of London, 2007-2009).

### 1.1.3 Photosynthetic pigments. Chlorophylls and carotenoids

As outlined before, two main classes of pigments are responsible for light absorption, charge separation and energy transfer toward the RC in both photosystems: chlorophylls and carotenoids. The different photosynthetic pigments can be distinguished by their absorption spectra (Figure 2A).

#### Chlorophylls

The basic component of all different types of chlorophylls is a porphyrin (a cyclic tetrapyrrole) in which the four nitrogen atoms of the pyrroles coordinate a magnesium atom. A fifth ring and the phytyl, a chain of 20 carbon atoms responsible for their hydrophobicity, are also present. Different chlorophylls are distinguished from their substitutions: in higher plants, two types of molecules are present, differing in a substituent in the second pyrrhole ring: a methyl for the chlorophylls *a* (Chl *a*), an aldehyde for the chlorophylls *b* (Chl *b*) (Figure 2B). The characteristic ability of chlorophylls to absorb light in the visible region is due to the high number of conjugated double bonds present in these molecules (Figure 2B).

LCHII is the main thylakoid component accounting for more than half the total chlorophyll and intrinsic protein of most plant thylakoids (Thornber, 1986). Plants and green algae have a mobile pool of chlorophyll a/b-binding proteins that can switch between being light harvesting antenna for PSI or PSII, in order to maintain an optimal excitation balance (Kargul and Barber, 2008). The marked adaptation of the pigment composition and content of higher plant thylakoids from plants grown under different light intensities is well established. Obligate shade plants have much more chlorophyll per chloroplast and lower Chl*a*/Chl*b* ratios (~2.0 – 2.4) compared to sun plants (~2.8 – 3.6) (Anderson, 1986).

In *C. reinhardtii*, the core complex of PSII contains between 40 and 50 Chl, with a Chl *a*/Chl *b* ratio >14 (Hobe et al., 2003).

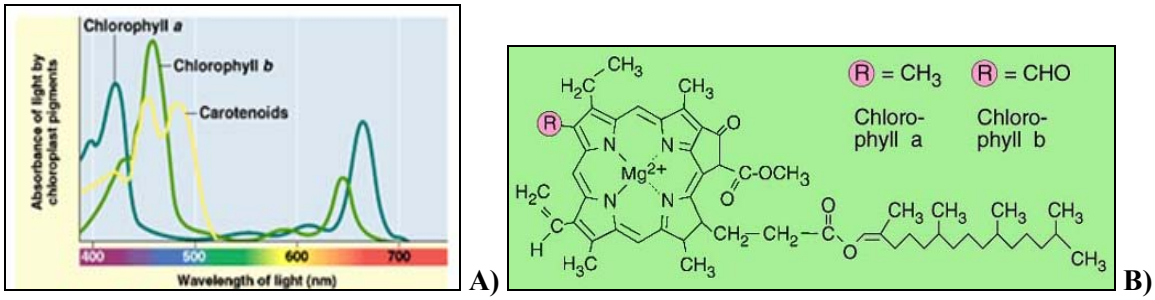



**Figure 2. Chlorophylls. A**) Absorption spectra of chlorophylls. **B**) Carotenoids structure of chlorophyll *a* and *b*.

## Carotenoids

Carotenoids are among the most widespread natural pigments and fulfil a variety of functions, playing essential roles in organisms performing oxygenic photosynthesis. Carotenoids are polyisoprenoid compounds containing 40 carbon atoms. They possess a long chain of conjugated double bonds in the central part of the molecule, and variable end groups: different level of hydrogenation and introduction of oxygen-containing functional groups create a large family of over 600 natural compounds (Green and Durnford, 1996). In higher plants, two different classes are found into thylakoids: (i) carotenes (e.g. β-carotene), which are hydrocarbones with linear structure and with cyclic groups in one or both extremities, and (ii) xanthophylls (e.g. lutein, zeaxanthin), which are oxygenated derivatives of the first group.

In higher plants, the carotenoids normally associated with thylakoid membranes are α- and β-carotene (bound especially to the core complex of both photosystems) (Green and Durnford, 1996) and the xanthophylls lutein, zeaxanthin, violaxanthin and neoxanthin (bound to antenna complexes) (Formaggio et al., 2001; Caffarri et al., 2001). Carotenoids are non-covalently

bound to the protein complexes, probably involving hydrophobic interactions (Gastaldelli et al., 2003).

Carotenoids have at least three main roles in photosynthesis: a) structure stabilisation and assembly of protein complexes in the thylakoid membrane; b) light absorption and excited state energy transfer to the chlorophylls (Green and Durnford, 1996); c) protection against photo-oxidative damages (Havaux and Niyogi, 1999).

Carotenoids composition of thylakoid is not constant: it can undergo modifications during long-term acclimation of plants to stressing condition, as well as rapidly changes following fluctuations of solar light intensity (Green and Durnford, 1996). The xanthophyll cycle (Figure 3) involves the three xanthophylls violaxanthin, antheraxanthin and zeaxanthin, and consists in a light-dependent, reversible de-epoxidation of violaxanthin to zeaxanthin *via* the intermediate antheraxanthin; the former reaction is catalysed by violaxanthin deepoxidase (VDE), a lumenal enzyme activated by acidification of the luminal compartment (Green and Durnford, 1996).

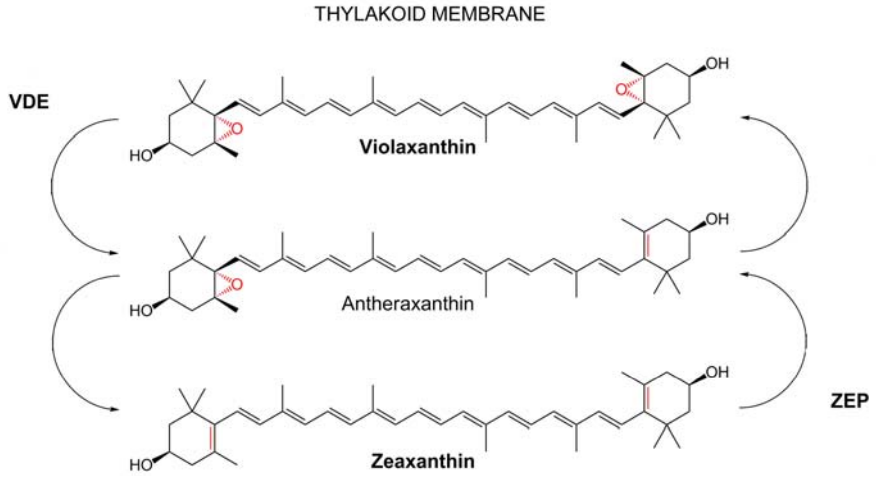



**Figure 3. Biosynthetic pathway of β-carotene-derived xanthophylls in higher plants**. The arrows between pigments denote enzymatic conversion caused by xanthophyll cycling. Enzymes involved are also reported.

Carotenoids involved in the xanthophyll cycle are localized in the peripheral antenna proteins of PSII (Ruban et al., 1999), not in the core complex and inner antenna of PSII. Upon de-epoxidation, the newly synthesized zeaxanthin is distributed among LHCII and minor antennae.

### 1.1.4 Carotenoids and plastoquinone biosynthesis

In vascular plants and algae carotenoids are usually confined to the plastid, which is also the site of their synthesis. Most carotenoids are bound to membrane proteins associated with the

photosynthetic apparatus, and to some extent with plastid envelope membranes (Derguini et al., 1991). Among green algae, the ability to accumulate extraplastidic carotenoids under unfavourable conditions is frequently encountered. For example, astaxanthin of the green alga *Haematococcus pluvialis* is located in cytoplasmic lipid globules (Boussiba, 2000). The prominent carotenoids in green algae and vascular plants are *β*-carotene, lutein, 9-*cis*-neoxanthin, and violaxanthin (Figure 4), being rapidly converted to antheraxanthin and zeaxanthin under high light conditions, as reported above.

The carotenogenic enzymes are encoded by nuclear genes in *C. reinhardtii*. Biosynthesis of carotenoids (Figure 4) is initiated with the formation of isopentenyl-diphosphate (IPP), called "active isoprene." The subsequent reaction sequences are often divided into four stages: (*a*) stepwise condensation of isoprene units to form the first carotenoid, phytoene, (*b*) extension of the $\pi$-electron system by sequential desaturation resulting in lycopene formation, (*c*) cyclization reactions that generate the carotenes, and (*d*) synthesis of xanthophylls by the introduction of the oxygen functions (Grossman et al., 2004). Sequential addition of three molecules of IPP, results in the formation of the C20-compound geranylgeranyl pyrophosphate (GGPP) (Figure 4). This chain elongation is catalyzed by the enzyme GGPP synthase. GGPP is not only an intermediate in carotenogenesis, but also a substrate for the formation of a variety of other cellular components, including the phytol moiety necessary for the synthesis of Chl, tocopherols and phylloquinone and, in the case of vascular plants but not algae or bacteria, the hormone gibberellin. In chloroplasts and mitochondria, polyprenyl transferases synthesize the long chain isoprenoid component of plastoquinone and ubiquinone, respectively, by adding isoprene units to GGPP (Figure 4).

Head-to-head condensation of two molecules of GGPP by phytoene synthase (PSY) results in formation of phytoene, the first carotenoid of the pathway. This reaction and all subsequent steps are linked to plastid membranes as the carotenoid substrates are hydrophobic and membrane associated (Schledz et al., 1996; Bonk et al., 1997). As PSY catalyzes the first committed step of carotenoid biosynthesis, it was expected to represent a key target for regulatory control. Experimental evidence supports this expectation. Over-expression of the *psy* gene in tomato plants resulted in dwarf plants because elevated phytoene production in this strain caused a severe reduction in gibberellin synthesis (Fray et al., 1995). Similarly, the carotenoid content of tomato fruits increased substantially in concert with the fruit-specific expression of a bacterial *psy* gene (Fraser et al., 2002). In vascular plants, *psy* mRNA increases in red light in a phytochrome-dependent manner (Von Lintig et al., 1997). In *H. pluvialis*, expression of *psy* appears to be under photosynthetic redox control because

increased transcript accumulation in high light could be prevented by the addition of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transport at PSII (Steinbrenner and Linden, 2001). In *C. reinhardtii*, *psy* mRNA increases following exposure of cells to blue light, suggesting that the gene may be under the control of a blue light photoreceptor such as PHOT1 or CRY1 (Bohne and Linden, 2002).

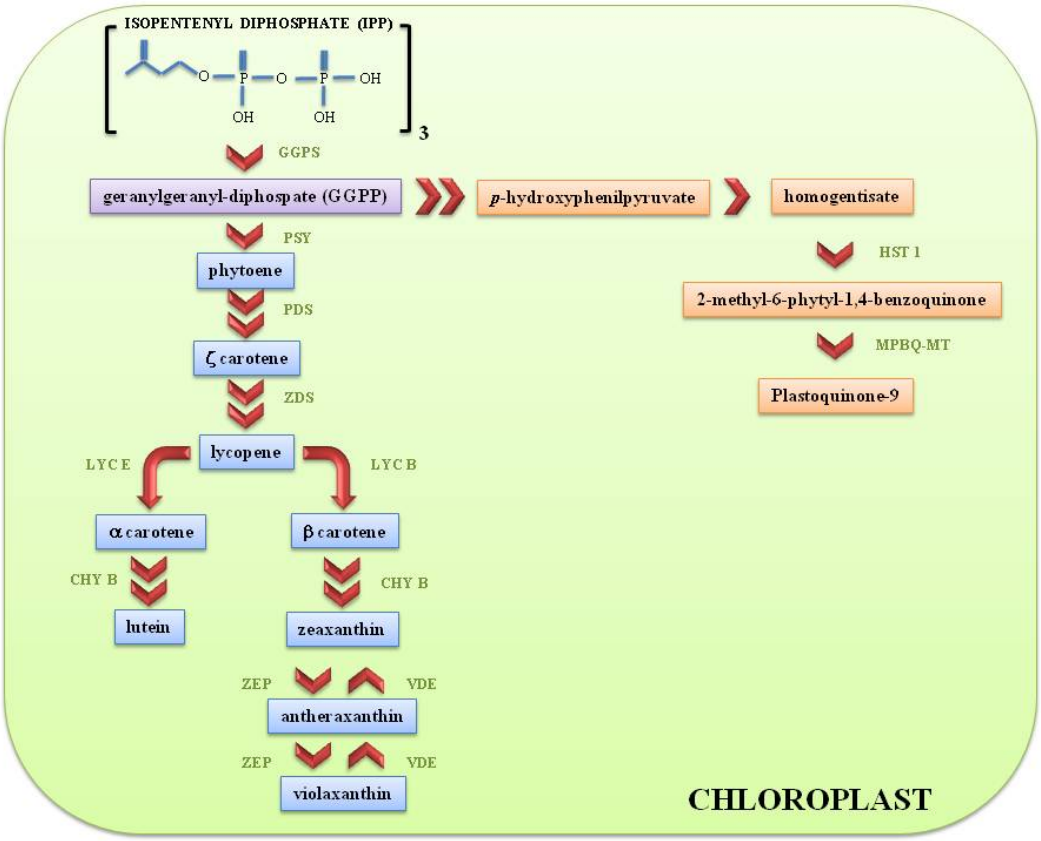



**Figure 4. Schematic representation of carotenoids (blue) and plastoquinone (red).** The carotenoid and plastoquinone biosynthetic pathways in *Chlamydomonas reinhardtii* chloroplast. In green the mainly enzymes are reported too.

Phytoene contains only three conjugated double bonds and consequently shows no visible absorbance. In vascular plants and green algae, two desaturases sequentially introduce additional conjugated double bonds into phytoene, thereby extending the $\pi$-electron system and shifting the absorbance to longer wavelengths. These enzymes are structurally and functionally related and likely have evolved from a common ancestor by gene duplication (Sandmann, 2002). The initial desaturation by phytoene desaturase (PDS) introduces two double bonds into the molecule, resulting in the formation of $\zeta$ -carotene. Two additional desaturation steps catalyzed by $\zeta$ -carotene desaturase (ZDS) yield lycopene. Both PDS and

ZDS contain an amino-terminal conserved dinucleotide binding motif and use FAD as a redox cofactor. Electrons generated by the reaction are transferred from $FADH_2$ to plastoquinone, and from there can be funnelled either into the photosynthetic electron transfer chain or to $O_2$ via a plastid terminal oxidase (Carol and Kuntz, 2001). Similar to *psy*, expression of *pds* is under photosynthetic redox control in *H. pluvialis* (Steinbrenner and Linden, 2003), but is induced by blue light in *C. reinhardtii* (Bohne and Linden, 2002).

Following cyclization of lycopene the carotenogenesis pathway splits, forming the *α*- and *β*-branches of carotenes (Figure 4). Introduction of *β*-ionon rings at both ends of the linear lycopene molecule results in the formation of the symmetric *β*,*β*-carotene, termed *β*-carotene. This reaction is catalyzed by a single enzyme, lycopene *β*-cyclase (LCYB). The concerted action of LCYB on one side of the lycopene molecule and a second cyclase on the opposite end results in formation of *β*,*ε*-carotene, more commonly known as *α*-carotene. In most plants, the enzymatic capacity of the second cyclase, designated lycopene *ε*-cyclase (LCYE), is restricted to the introduction of a single *ε*-ionon ring per lycopene (Cunningham and Gantt, 1998). The *α*-carotene molecule is the precursor of lutein and loroxanthin, whereas violaxanthin, zeaxanthin, and neoxanthin are synthesized from *β*-carotene. The genes encoding both *lyc-β* and *lyc-ε* are nuclear and have been cloned from a number of vascular plants (Cunningham et al., 1996; Hirschberg, 1998). The encoded proteins share significant similarity and the genes probably evolved as a consequence of a duplication (Cunningham and Gantt, 2001; Sandmann, 2002).

In photosynthetic tissues of vascular plants, the introduction of oxygen functions into carotenoids is limited to hydroxylations and epoxidations. Hydroxylation takes place at the C3-atoms of the ionon rings of both α- and β-carotene, resulting in the formation of lutein (β,ε-carotene-3,3-diol) or zeaxanthin (β,β-carotene-3,3-diol), respectively. The β-ionons can be oxidized by carotene β-hydroxylase (CHYB), a nonheme di-iron protein with four predicted trans-membrane helices (Bouvier et al., 1998; Sun et al., 1996). *H. pluvialis* is the only green alga from which a putative *chy-β* homolog was cloned, and the encoded protein was shown to be capable of hydroxylation of *β*-ionon rings as well as their 4-keto derivatives (Linden, 1999). Like the other carotenogenic genes, expression of *chy-β* from *H. pluvialis* is under redox control (Steinbrenner and Linden, 2001; Steinbrenner and Linden, 2003). Violaxanthin is synthesized from zeaxanthin *via* the intermediate antheraxanthin by the stepwise introduction of epoxy-groups at the C5, C6 double bonds of the two ionon rings. Epoxidation by *zep* is restricted to *β*-ionon rings carrying a hydroxyl group at the C3 position (Bouvier et al., 1996). The *zep* gene from *C. reinhardtii* was identified by searching an EST

library for clones encoding polypeptides with similarity to vascular plant *zep*; strains of this alga with point mutations in the *zep* gene were impaired in zeaxanthin epoxidation (Baroli et al., 2003). In vascular plants, violaxanthin de-epoxidase (VDE) catalyzes the reverse reaction of the ZEP enzyme, the de-epoxidation of violaxanthin to form zeaxanthin via antheraxanthin. Although not necessary for carotenoid biosynthesis, the reaction is part of the photoprotective xanthophyll cycle involved in the generation of hydroxy-carotenoids that function in the dissipation of excess absorbed light energy within the light-harvesting apparatus (Niyogi, 1999). The *vde* gene was identified in a number of vascular plants (Bugos et al., 1998; Bugos and Yamamoto, 1996; Emanuelsson, 2003; Zhang et al., 2003), and a VDE mutant of *C. reinhardtii* (*npq1*) was isolated by screening for mutants with reduced non-photochemical quenching (NPQ) (Niyogi et al., 1997). Loroxanthin, a xanthophyll present in *C. reinhardtii* but not in vascular plants, is derived from lutein by hydroxylation of the methyl group at C9 of the polyene chain (Britton, 1998). The mechanism of this reaction and the nature of the putative loroxanthin synthase (LSY) are not known.

Surprisingly, although there are no reports on the presence of astaxanthin in *C. reinhardtii*, its genome contains an open reading frame with strong similarity to the carotene *β*-ketolase (BKT) from *H. pluvialis*; this enzyme catalyzes the introduction of keto groups at C4 and C4 of *β*-carotene as a step toward the biosynthesis of astaxanthin (Britton, 1998; Lotan and Hirschberg, 1995). This putative *bkt* gene is located next to *chy-β* and expressed since it is represented in cDNA libraries. A biochemical analysis of the protein product of this gene will establish whether it has ketolase activity or has acquired a different catalytic function.

### 1.1.5 The PSII D1 core protein

As described above, the scaffold of the PSII reaction centre is formed by two protein subunits, D1 and D2, each composed of five transmembrane α-helices (named from A to E) (Satoh, 1993) with their N- and C-termini exposed to the stromal and lumenal sides, respectively (Xiong et al., 1996). Moreover, other two helices on the stromal (in DE loop, between helices IV and V) and lumenal side (in CE loop, between III and IV helices) are present (Figure 5). The D1 protein acts both as a structural and multifunctional component of the reaction centre, mediating both photosynthetic electron transport and oxygen evolution (Aro et al., 2005; Barber, 2006).

D1 is synthesized as a 33.5–34 kD precursor on chloroplast ribosomes anchored on the non-appressed stromal lamellae and processed at the carboxy terminus (Edelman and Mattoo,

2008) as a part of a maturation process that is required for the assembly of the manganese-cluster into PSII (Diner et al., 1988; Satoh and Yamamoto, 2007).

Mature D1 is assembled into a native PSII complex on the stromal lamellae (Ghirardi et al., 1990, 1993) and then translocated to grana lamellae, where it becomes functional (Mattoo and Edelman, 1987).

Due to continuous damage in the light through inevitable generation of harmful reactive oxygen species, D1 is continuously degraded and re-synthesized to maintain photosynthetic electron transport, making D1 the protein with the highest turnover rate in the chloroplast (Mattoo et al., 1984; Jansen et al., 1999; Kanervo et al., 2005). After damage, the D1 protein is promptly degraded by proteolytic enzymes or forms specific aggregates with nearby polypeptides such as the D2 or CP43 protein (Aro et al., 1993; Yamamoto, 2001). Two families of proteases are involved in catabolism of mature D1 protein, which are the ATP-independent serine endoprotease Deg/Htr family (Huesgen et al., 2005) and the ATP-dependent zinc metalloprotease FtsH family (filamentation temperature sensitive H) (Adam et al., 2006).

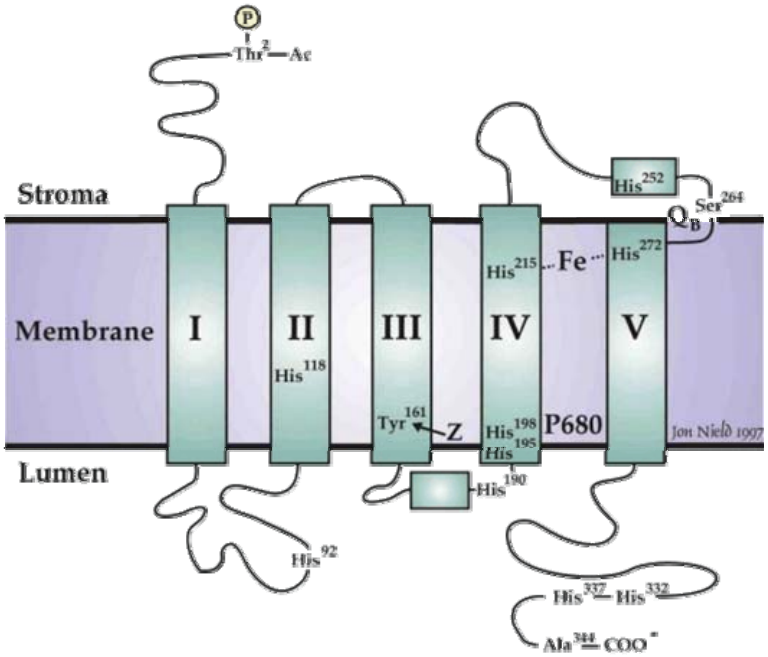



**Figure 5. The membrane folding pattern of mature spinach D1 protein**. Roman numerals I-V indicate the membrane-spanning helices (A-E). Two minor helices, on the stromal and lumenal sides, are reported. The putative positions of the bound cofactors and histidine residues on the proposed trans-membrane helices are shown. Sequence data were obtained from SWISSPROT database.

### 1.1.6 Effect of stressful light and temperature conditions on thylakoid structure

Light and temperature stressful conditions determine significant modifications in the structures of thylakoids and PSII complexes, resulting in photosynthesis impairment. High light and high temperature have an evident effect on the PSII activity. During the heat stress, the $Q_B$ plastoquinone is destabilized and released from its binding pocket. A conformational modification was expected to take place around the $Q_B$ site in the DE-loop of the D1 protein, but the exact nature of the structural change is not known. Simple physical perturbation by heat of the stroma-exposed DE-loop is possible, but another possibility is that the DE-loop was chemically modified from the temperature stress, which leads to the conformational change in the loop structure (Yamamoto et al., 2008).

It was also shown that the oxidation rate of the reduced primary electron acceptor of PSII is significantly affected by the fluidity of the thylakoid membranes, suggesting the importance of lateral diffusion of the lipids in the functioning of the acceptor side of PSII (Yamamoto et al., 1981). If the fatty acids of the lipid around the $Q_B$ site are unsaturated, moderate heat stress may cause lipid peroxidation which may subsequently damage the DE-loop of the D1 protein. Complete recovery of the heat-damaged PSII was not observed, probably because the release of $Q_B$ plastoquinone at the acceptor-side of PSII and of the extrinsic proteins and Mn at the donor-side are irreversible processes. At higher temperatures, the repair system including the proteases is also damaged, this could be the reason for the irreversible inhibition of PSII (Murata et al., 2007).

Thylakoid membrane un-stacking is a stress-induced phenomenon related to structural modification of lipids and proteins assembly (Figure 6).

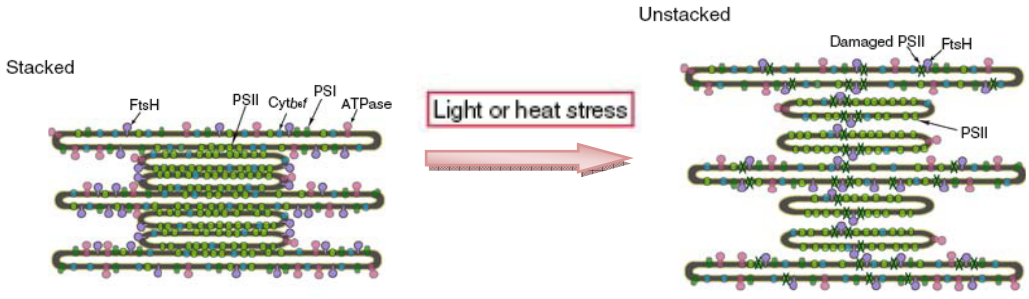



**Figure 6. A model of thylakoid un-stacking under light or heat stress.** PSII, PSI, cytochrome b6f, ATPase, and FtsH complexes are shown by symbols with pale green, dark green, blue, red and purple colour, respectively. Un-stacking of the thylakoids was depicted in an exaggerated manner. Actually stress-induced un-stacking takes place only partially and the stacked membrane region which is resistant to the stresses exists as a ‘‘grana core’’ (Adapted from Yamamoto et al., 2008).

It has been shown that thylakoid stacking is regulated by electrostatic interactions on the membrane surfaces, which is determined by the distribution of surface electric charge over the membranes (Barber, 1982). LHCII seems to be crucial for thylakoid stacking. It is well known that under long-term light stress, LHCII decreases its antenna size. Destabilization of chlorophylls and degradation of the apoproteins could occur during this process (García-Lorenzo et al., 2005).The irreparable consequence of the stress-induced un-stacking of the thylakoids assayed *in vitro* suggests that the surface properties of the thylakoid membranes changed drastically and irreversibly during the stress. Probably important for the irreversible membrane un-stacking are the changes in the distribution of protein complexes such as LHCII in the thylakoids as well as the structural changes of the individual proteins in PSII and changes in the lipid environments of the thylakoids.

It is well established that the oxidation of water and reduction of plastoquinone during the light reactions of photosynthesis cause an accumulation within PSII of oxidizing radicals, including reactive oxygen species (ROS) (Powles, 1984; Asada, 1996; Foyer and Noctor, 2003; Apel and Hirt, 2004).

In detail, the rate of ROS accumulation in PSII increases with light intensity (Hirayama et al., 1995) and was linked by many investigators to photoinhibition (Adir et al., 2003), "a light-dependent irreversible inactivation of PSII reaction centre activity, which can be restored only *via* the degradation and synthesis of the Dl protein'' (Tyystjärvi and Aro, 1996). Photoinhibition reduces the photosynthetic capacity, when absorbed light energy exceeds the ability of the organism to repair the photodamage by *ex novo* synthesis of PSII proteins.

Two mechanisms have been proposed to account for the photoinhibition of PSII, namely acceptor-side and donor-side photoinhibition (Figure 7) (Barber and Andersson, 1992; Aro et al., 1993; Yamamoto, 2001).

Damage to the oxidizing side of the PSII reaction centre *via* impaired electron donation from the oxygen-evolving complex (Callahan et al., 1986), and/or damage to the reducing side *via* blocked electron flow from $Q_A$ to $Q_B$ (Vass et al., 1992), was singularly, or sequentially (Song et al., 2006), implicated in eliciting ROS (Figure 7). PSII-derived ROS were theorized to trigger D1 protein degradation by changing the conformation of the protein and rendering it susceptible to protease (Aro et al., 1993). ROS were also suggested to act directly on the D1 protein, oxidizing amino acids close to the redox active components of PSII (Sharma et al., 1997). The idea that ROS may be involved in D1 degradation was based on evidence that scavengers of oxygen-free radicals inhibit light-dependent degradation of the D1 protein while increasing the photosynthetic efficiency of Spirodela plants (Sopory et al., 1990).

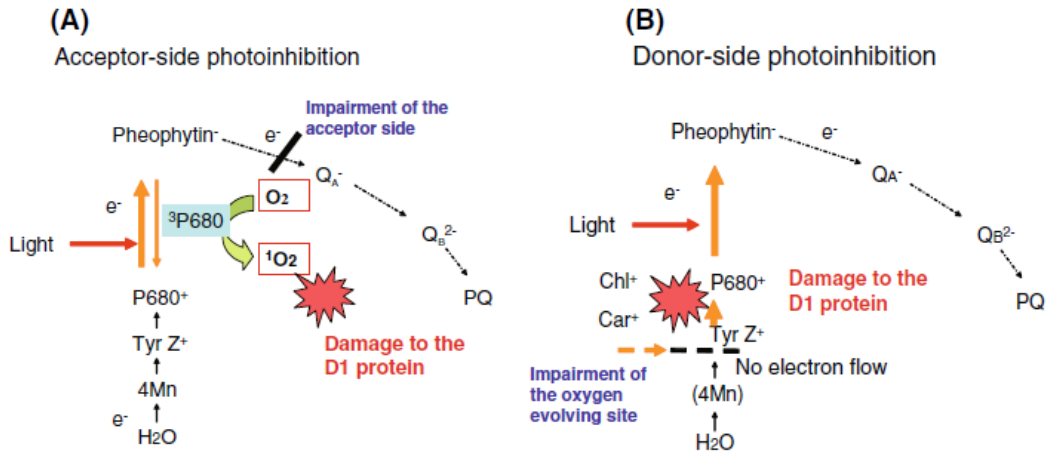



**Figure 7. Mechanism of the photoinhibition of PSII. (A)** The acceptor-side photoinhibition of PSII. **(B)** The donor-side photoinhibition of PSII. In the acceptor-side photoinhibition, excess illumination with visible light induces $^1O_2$, which damages the D1 protein, while in the donor-side photoinhibition, the cationic radicals formed at the donor-side of PSII by the illumination damage the D1 protein (Yamamoto et al., 2008).

### 1.1.7 Adaptation response of photosynthetic apparatus to high light condition

Photosynthetic organisms evolved a particular sensitivity to light changes in the environment. Under high illumination, when light is a factor in excess, the turnover rate of the reaction centres and electron transport speed are rate limiting. In these conditions, excess excitation energy from absorbed photons cannot be used for the oxidation of water (photoinhibition) and the oxidized intermediates, generated through light absorption, may led to close bio-molecules damaging. To reduce photodamage, photosynthetic organisms dynamically regulate light-harvesting and carbon fixation to balance the absorption and light utilization via reversible short- and long-term physiological responses referred to as photo-acclimatation. Hence, in order to assure optimal efficiency of photosynthesis under both strong and weak illumination, protecting mechanisms must be up and down regulated.

Algae and plants have evolved non-photochemical quenching (NPQ) mechanisms to dissipate excess absorbed light energy as heat (Horton et al., 1996; Gilmore, 1997; Müller et al., 2001; Holt et al., 2004). NPQ, measured as non-photochemical quenching of chlorophyll *a* (Chl *a*) fluorescence, consists of at least three components (Horton and Hague 1988; Müller et al., 2001). The main element, called qE, reflects the de-excitation of singlet excited Chl ($^1$Chl*) in the PSII antenna by a feedback regulatory system that involves: (i) an increase of proton gradient (ΔpH) across the thylakoid membrane by an accelerate photosynthetic electron transport due to a higher sunlight absorption than $CO_2$ fixation plant's capacity; (ii) a

synthesis of high-light-induced xanthophylls such as zeaxanthin and (iii) of the LHCII superfamily proteins (Müller et al., 2001). Indeed, when light absorption exceeds photosynthetic capacity, the degree of the ΔpH increases due to a reduction of $H^+$ conductance ATP synthase (Kanazawa and Kramer, 2002). The decrease in lumen pH in excessive light activates the inter-conversion of specific xanthophyll pigments that are mostly bound to LHC proteins. A low pH in the thylakoid lumen activates violaxanthin de-epoxidase (VDE), which converts violaxanthin to antheraxanthin and zeaxanthin via the xanthophyll cycle (Yamamoto et al., 1962) (Figure 1). This enzyme has a pH optimum of approximately 5.2, suggesting that it is located in the thylakoid lumen, which becomes acidic as the cells photosynthesize (Yamamoto et al., 1999). Lumen acidification facilitates attachment of the enzyme to the thylakoid membranes and access to violaxanthin (Hager and Holocher, 1994), its membrane-associated substrate. A different enzyme, zeaxanthin epoxidase (ZEP), catalyzes the epoxidation reactions that complete the violaxanthin cycle. Because of its pH optimum of 8, ZEP is thought to be located on the stromal side of the thylakoid membrane and to be constitutively active (Müller et al., 2001). The level of zeaxanthin is therefore determined by the activity of VDE and ZEP. Moreover, several LHC proteins, associated with PSII have been implicated in qE. In particular the minor LHC proteins CP29 and CP26 were suggested to be involved in qE based on the relative enrichment of associated xanthophyll cycle pigments (Bassi et al., 1997). The ΔpH mediated induction or reversal of qE is very quick, enough to cope with natural light intensity modifications, occurring within seconds to minutes of a variations in light intensity.

Mutants affecting in $H^+$ translocation, by electron transport of the cytochrome $b_6/f$ complex (Munekage et al., 2001), to plastocyanin (Shikanai et al., 2003), and PS I cyclic (Munekage et al., 2002), limit acidification of the thylakoid lumen and finally inhibit qE. Mutants that are deficient in qE (called npq mutants) were isolated first in *C. reinhardtii* and later in *Arabidopsis thaliana* by using video imaging of Chl fluorescence quenching (Niyogi et al., 1997, 1998; Shikanai et al., 1999; Peterson and Havir, 2000).

The zeaxanthin implication in qE was demonstrated by a strong correlation between zeaxanthin concentration and qE in leaves (Demmig-Adams et al., 1990) and thylakoids (Gilmore and Yamamoto, 1991), by inhibition of VDE and by analysis of *C. reinhardtii* and *A. thaliana* mutants, that are defective in the xanthophylls cycle (Niyogi et al., 1997, 1998). In particular, zeaxanthin, is a key component in the activation of thermal dissipation of excitation energy in excess (Havaux and Niyogi, 1999; Holt and al., 2005).

In tobacco seedlings (*Nicotiana tabacum* L. cv Samsun) was observed a different transcript expression levels between *zep* and *vde* genes under low and moderate light conditions. In particular, high transcript levels of *zep* and *vde* and relative pigments production were found in early stages of chloroplast development after 5 hours of illumination with moderate light intensities. These data indicated that also moderate light intensities are sufficient to induce lumen acidification hence activation of the VDE enzyme (Woitsch and Romer, 2003).
Recently, it was demonstrated that heat induces isomerization of violaxanthin and zeaxanthin (Niedzwiedzki et al., 2005; Milanowska and Gruszecki, 2005).

**1.2 Correlation between human health and photosynthetic compounds**

Human health is determined by the interaction of several factors such as genetic traits, eating-habits and life-styles that in turn, are all influenced by the environment and its changes. Currently, several disciplines are bringing to light the complex pathways by which environmental factors influence health patterns. Environmental changes arising from urbanization, population increases, industrial and agricultural activities have resulted in thermal fluctuations, reduced air, water and soil quality, and increased exposure to radiation and persistent chemical pollutants that can trigger various disease processes. In particular, environmental degradation and chemical overload coming from agriculture practices have been linked to diseases such as respiratory and cardiovascular problems, neurological and physiological disorders, and increased incidence of cancers (Valko et al., 2007). In addition to the built environment, several life style choices such as smoking habits, decrease of physical activity and consumption of alcohol and drugs can have profound impacts on our health and have been associated with the above mentioned pathologies. Among lifestyles, dietary factors greatly affect human well-being. Poor eating-habits leading to inadequate intake of calories can negatively affect our health and have been unequivocally and causally associated with the risk of obesity, cardiovascular disease, type 2 diabetes, stroke, cancers and neurodegenerative disorders (Mattson, 2008).
Another harmful health risk factor common to the development of these and other chronic pathologies (Maritim et al., 2003; Andersen, 2004; Singh and Jialal, 2006; Ward and Croft, 2006; Bez and Yau, 2008; Pacher and Szabo, 2008; Manos et al., 2009) is oxidative stress (Figure 8), a process resulting from an imbalance between excessive production of reactive oxygen species and/or nitrogen species and limited action of antioxidant defences (Spitz et al., 2004). As oxidative stress is believed to play a major role in the ageing process and in several diseases, there is considerable public interest in the antioxidative effects of dietary factors

(Lesgards et al., 2002; Mattson, 2008). Indeed, with proper nourishment, the body can, on its own, make sufficient quantities of antioxidant enzymes and substrates for those enzymes. These can facilitate the quenching of excess free radicals by antioxidants. An enhancement in dietary intake of antioxidants and phytochemicals with related functions can counteract oxidizing species and potentially restore a healthy cellular redox balance (Vattem et al., 2005).

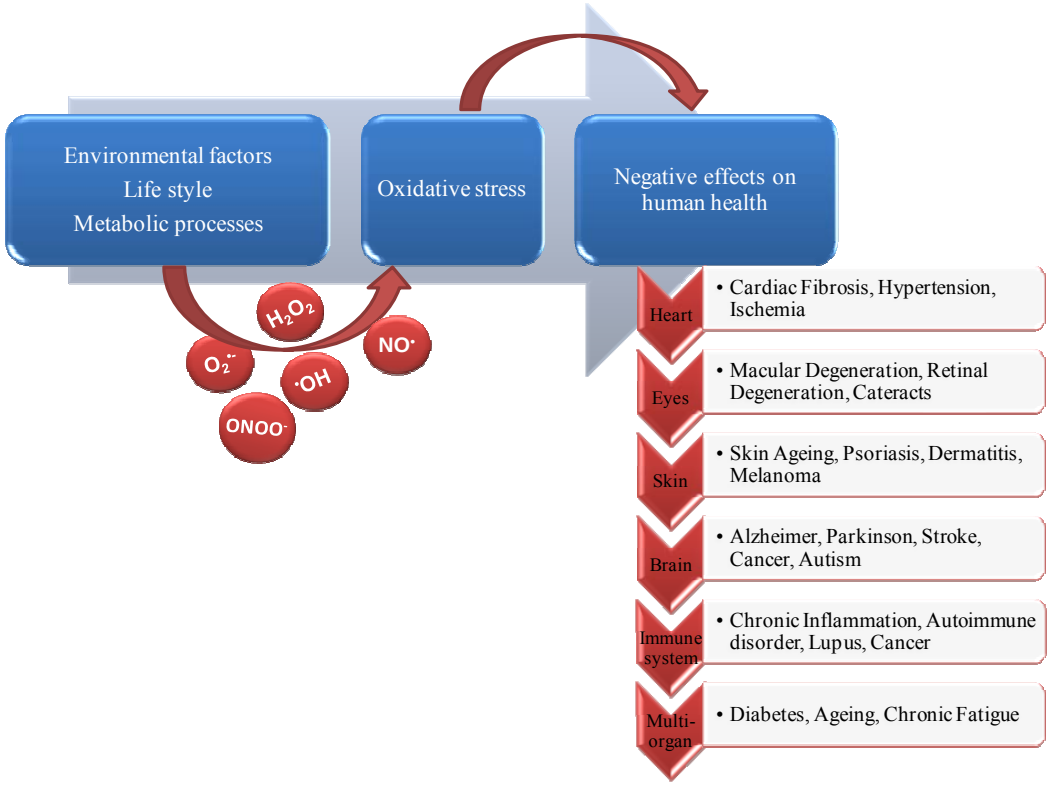



**Figure 8. The main factors and protagonists in the development of oxidative stress, and the possible negative outcomes to human health.**

However, in recent years, following the achievement of the human genome sequencing, the exploitation of molecular biology tools revealed that specific antioxidant compounds modulate various cellular functions, acting not only as radical scavengers, but also as regulators of genes and enzymes expression. This approach gives rise to the new sciences of nutrigenomics and nutrigenetics which consider the relationship between specific nutrient or diet and gene expression and determine how genetic variability affect the response to diet, respectively (Knasmüller et al., 2008). In this scenario, nutraceutics, which are food or part of a food providing medical or health benefits, acquire a new additional significance for the development of novel food and personalized diets.

Nowadays, the consumption of midday and/or midnight snack food is becoming a dominant eating-habit, despite its close correlation with several metabolic disorders. Eating small amounts of food on a regular basis is considered healthy, but often snacks available in the market provide excess calories and fats and little or no nutrient value to the diet. Moreover, most snacks contain preservatives, sweeteners and flavouring that can have negative effects on health.

There is no doubt that a healthy diet must include proper food selection, keeping track of meal times and regulation of the amount of food intake. The limits imposed by modern life-styles on eating-habits create a necessity to develop an adequate food management rather than simply body-weight control. One of the most popular eating diet plans suggests the 40:30:30 ratios for carbohydrates, proteins and fats that a person should consume. With this ratio of components, the body is able to balance insulin and glucagon, as well as to realize a more effective internal metabolism. Currently, experts from the manufacturing sector are concerned with the development of modern alimentary strategies aimed at those who believe in health through correct nutrition and food supplementation.

### 1.2.1 Applications of plant-derived phytochemicals

Plants produce a big number of chemicals that are important for their function and development. Some of these compounds are primary metabolites, which include proteins (aminoacids), carbohydrates, fats, nucleic acids etc. Besides these primary chemicals, the plants also produce secondary metabolites, which are specific to some taxonomic groups (families, genera) (Krzyzanowska et al., 2009).

Compared to the main and most abundant molecules found in plants, these secondary metabolites are defined by their low abundance, often less than 1-5% of the dry weight. They are formed under environmental pressure and have no recognized role in the maintenance of fundamental life processes in plants; but, they contribute mainly to protect plants against several environmental stresses. This group includes classes of compounds able to promote public health, with different biological activities (Table 1), which in traditional medicine were used for centuries to cure or protect from diseases.

A correlation between diet and health has been known since ancient time. However, numerous epidemiological studies confirm the correlation, especially regarding the frequency of some diseases incidence in relation to the intake of particular nutrients. In particular, a low consumption of fruit, vegetables and whole grain was often associated to disease.

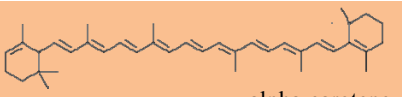
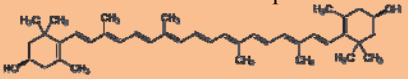
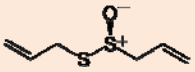
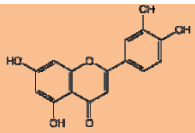
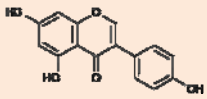
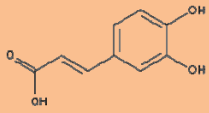
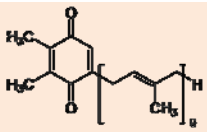
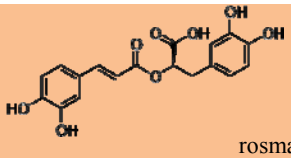
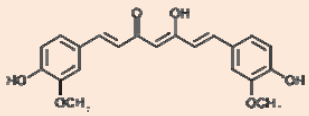
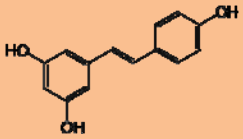
| ACTIVE METABOLITE *(dietary sources)* | STRUCTURE | MAIN NUTRACEUTIC ACTIVITY |
|---|---|---|
| Carotenoids [1] *(carrot, tomato, spinach, broccoli, kale)* |  alpha-carotene  zeaxanthin | antioxidant, anticancer, immunostimulation, vision, protection |
| Cysteine derivates [2, 3] *(garlic, onion)* |  allicin | antioxidant, antibacterial, anti–arteriosclerosis, anticancer, antibiotic, lipid lowering |
| Flavones [4,5] *(cereal, herbs, citrus fruit, tea)* |  luteolin | antioxidant, antibacterial, anticancer, anticolitis |
| Isoflavones [6] *(pea, bean, soybean, salvia)* |  genistein | antioxidant, anti -arteriosclerosis |
| Phenolic compounds [7] *(cichorium, coffee, lettuce, red wine)* |  caffeic acid | antioxidant, cardio protective, antihypertensive |
| Plastoquinones [8] *(green vegetables and fruits)* |  plastoquinone | antioxidant, anticancer |
| Polyphenolic acids [9] *(rosemary, sage, oregano, basil, lemon balm)* |  rosmarinic acid | antioxidant, antibacterial |
| Ferulic acids and derivatives [10] *(apple, orange, peanut, pineapple)* |  curcumin | antioxidant, cardio protective, immunostimulation |
| Phytoalexin [11, 12] *(grapes, pine nuts, peanuts, wine)* |  resveratrol | antioxidant, cardio protective, anti-arteriosclerosis, anticancer |

**Table 1**. **Secondary metabolites, dietary sources, structures and related functional activities.**

([1]Mares-Perlman et al., 2002, [2]Cai et al., 2007, [3]Ariga and Seki, 2006, [4]Fiamegos et al., 2004, [5]Cermak, 2008, [6]Romani et al., 2003, [7]Vagi et al., 2005, [8]Kruk et al., 2006, [9]Tepe, 2008, [10]Yongyue et al., 2005, [11]Prokop et al., 2006, [12]Baur and Sinclair, 2006).

Several studies indicate that populations consuming high levels of plant derived foods, namely fruits-including berries, nuts, vegetables, whole-grains, legumes, seeds, various types of tea and a bewildering array of spices, but also low in red meat and animal fats, have low incidence rates of various cancers (Krzyzanowska et al., 2009; Whale et al., 2009).

Such diets are exemplified by the classical Mediterranean diet of Southern Europe and the highly vegetarian diets of South East Asian populations (Block et al., 1992; Messina et al., 1994).

The research for natural, plant-derived phytochemicals that express anticancer properties, is not restricted to food plants. Plant secondary metabolites are a very important industrial and economic source of many drugs, flavors, insecticides, fragrances and dyes. Pharmaceutical companies are currently isolating and assessing the anticancer potential of numerous phytochemicals from all regions of the world, in order to discover more efficient treatments for various types of cancer (Krzyzanowska et al., 2009).

The technology of large-scale plant cell culture is feasible for the industrial production of plant-derived fine chemicals, but there are still several limitations.

The natural content of the valuable compounds in a plant is often very low, or production is often impossible due to a lack of raw material. Some compounds can only be isolated from rare plants. On the other hand, chemical synthesis of these compounds is often not technically or commercially feasible because of their highly complex structures (Oksman-Caldentey and Inzé, 2004).

### 1.2.2 Carotenoid benefits for human health

Carotenoids are synthesised by plants, algae, fungi, yeasts and bacteria. Animals and humans cannot synthesise them and are dependent on the dietary sources (Fraser and Bramley, 2004; Stahl and Sies, 2005). Different 600 carotenoids have been identified in nature; however, only about 40 are present in a typical human diet. Human plasma and tissues contain only 20 carotenoids, which are represented mainly by β-carotene, lycopene, lutein, β-cryptoxanthin and α- carotene (Fraser and Bramley, 2004; Rao and Rao, 2007).

They are present in various types of food, but the major sources of dietary carotenoids include orange and yellow fruits and vegetables as well as green leafy vegetables. Smaller amounts can be extracted from milk and foods containing dairy fat, egg yolks, sea fish, and carotenoids added as colorants to foods during processing (Rock, 1997).

Carotenoids are important components of the human diet because they have been linked to a multitude of health benefits. They play an important role in the cell communication and

protection against photo-oxidative processes by acting as singlet molecular oxygen, as well as, peroxyl radical scavengers and can interact synergistically with other antioxidants (Reddy et al., 2003). In mammals, some of them can be metabolised to retinol and function as vitamin A precursors. Positive effects between high dietary consumption and tissue concentration of carotenoids and reduced risk of chronic diseases such as age-related macular degeneration were also observed. There is also strong evidence showing that a rich diet in carotenoids prevents cardiovascular diseases and certain cancers like lung, colon, breast, and prostate cancer (Rao and Rao, 2007;Reddy et al., 2003; Palace et al., 1999).

Initially, most of this attention was directed towards β-carotene, also known as provitamin, the precursor of vitamin A, an essential nutrient for human health, responsible for the promotion of growth, cellular differentiation, morphogenesis, embryonic development, and visual function. Some carotenoids can be converted into active forms of this vitamin and, in doing so, may prevent vitamin A deficiency (Krzyzanowska et al., 2009).

Recently, the attention, is increased also for lycopene (the red pigment of tomatoes) as well as zeaxanthin and lutein (the yellow pigments responsible for the colour of corn and eggs). In the case of lycopene, there is some evidence for a protective role against prostate cancer (Ellinger et al., 2006). For lutein and zeaxanthin, considerable evidence (Mares-Perlman et al., 2002) is available for a role of these xanthophylls in the protection against age-related vision loss, such as age-related macular degeneration (AMD) (Seddon et al., 1994) and cataracts (Chasan-Taber et al., 1999; Brown et al., 1999). In addition, the important role for zeaxanthin and lutein in the protection against cancer and heart disease emerged (Mares-Perlman et al., 2002).

In particular, zeaxanthin and lutein are synthesized by plants (as well as photosynthetic microbes) and especially when these plants are grown in high light or under otherwise stressful conditions (Demmig-Adams and Adams, 1996; Demmig-Adams and Adams, 2006). Zeaxanthin, as mentioned above, protects against the formation of potentially destructive reactive oxygen species in leaves exposed to intense sunlight alone or moderate levels of sunlight in the presence of environmental conditions un-favourable for plant growth (Demmig-Adams and Adams, 2002). This photoprotective process is necessary for plant survival and reproductive success (Külheim et al., 2002); oxygenic photosynthesis could not exist without it. In addition, zeaxanthin serves in photoprotection *via* a second, not fully understood, mechanism that involves a direct inhibition of the oxidation of fatty acids of biological membranes (lipid peroxidation) (Havaux and Niyogi, 1999; Havaux et al., 2004).

Epidemiological studies have identified inverse links between zeaxanthin/lutein and a wide

range of human diseases (Mares-Perlman et al., 2002; Demmig-Adams and Adams, 2002; Sajilata et al., 2008). However, the underlying mechanisms for these apparent protective effects remain poorly understood.

Zeaxanthin/lutein protect the human eye from damage by intense light. A breakthrough in the understanding of the function of retinal zeaxanthin was made when it was shown that zeaxanthin prevents programmed cell death of retinal photoreceptor cells in an animal model (Thomson et al., 2002a; Thomson et al., 2002b). On the other hand, studies with human cancer cell lines provided evidence that lutein can stimulate programmed cell death of human breast cancer cells (Sumatran et al., 2000) and leukemic cells (Müller et al., 2002). Lutein furthermore selectively induces programmed cell death in mouse tumour cells, but decreases programmed cell death in cancer-fighting immune cells (blood leukocytes) of tumour-bearing mice (Chew et al., 2003). Up to now is not known how xanthophylls exert these remarkable and beneficial roles, including opposite effects on programmed cell death in different cell types.

For sure the zeaxanthin can modulate the oxidation of fatty acids (lipid peroxidation) in plants (Havaux and Niyogi, 1999; Havaux et al., 2004) as well as in humans (e.g. in epithelial cells of the eye's lens) (Chitchumroonchokchai et al., 2004); indeed, zeaxanthin protects lipids against destructive reactive oxygen *in vitro* (Wrona et al., 2003; Wrona et al., 2004).

As aforementioned, age-related blindness (with photoreceptor death) and cancer (with run-away cell proliferation) involve apparently opposite problems, i.e. either too little or too much programmed cell death. Current studies suggest that several dietary factors possess the capacity to ameliorate both of these contrasting conditions (Demming Adam et al., 2009). Thus far, phenolics (Youdim et al., 2002; Surh, 2003) the polyunsaturated fatty acids (of fish oil) (Seo et al., 2005), and zeaxanthin/lutein (Maccarrone et al., 2005) have all been shown to possess the notable ability to 'work both ways', i.e. triggering programmed cell death of unwanted cells while aiding in the survival of needed cells. This makes these food-derived compounds potentially highly desirable nutraceuticals.

As reviewed above, both zeaxanthin and lutein have roles in protecting human health. However, there is great disparity in how much zeaxanthin versus lutein can be obtained from leafy green plant foods. Indeed, the plant green parts typically contain high levels of lutein and maintain a few zeaxanthin amounts, because they carefully adjust the level of zeaxanthin in response to the light intensity. The level of zeaxanthin is finely controlled by a set of biochemical reactions, before mentioned. Human consumption of high levels of zeaxanthin is highly desirable, because zeaxanthin needs to be preferentially accumulated and incorporated

into the parts of the mammalian retina exposed to high irradiance (Landrum et al., 2001), and thus appears to be even more important in human diets than the more readily available lutein (Demming Adams et al., 2009). For this reason, arrest of the xanthophyll cycle in green leaves in the state of zeaxanthin may be a desirable trait to incorporate into crops that provide green leafy foods. Such a retention of zeaxanthin can be accomplished e.g. by knocking out or silencing the enzyme/gene (zeaxanthin epoxidase of the xanthophyll cycle) responsible for zeaxanthin conversion to violaxanthin and/or by overexpressing enzymes in earlier portions of the carotenoid biosynthetic pathway (Demming Adams et al., 2009).

As a generalization, over-expression of antioxidants in plants and algae may (i) make these organisms more resistant to abiotic stresses, while (ii) possibly increasing their susceptibility to some pests/pathogens, and (iii) likely also affecting growth, development, and reproduction. It may be feasible to simultaneously decrease crop losses due to environmental stress (like drought, heat, or frost), while enhancing the nutritional quality of food plants (Demming Adams et al., 2009).

Xanthophyll cycle mutants that accumulate zeaxanthin have already been produced in model plants and algae (Niyogi, 2000), and these traits can be transferred to crop plants. Due to the need for leaves to photosynthesize efficiently, overexpression of zeaxanthin in fruit, rather than leaves, is attractive. Tomato fruit with an increased zeaxanthin content has recently been engineered *via* two other manipulations (overexpression of lycopene-cyclase and beta-carotene hydroxylase) (Dharmapuri et al., 2002). Zeaxanthin-rich potato has also been produced (Romer et al., 2002) as well as zeaxanthin-accumulating *E. coli* (Albrecht et al., 1999).

### 1.2.3 Enrichment of nutraceutical contents in foodstuff

Molecular engineering of crop plants has offered a number of tools to markedly enhance intracellular concentrations of some of the beneficial nutrients. Some of these include increases in: protein level in potato; lysine in corn and rice; carotenoids (β-carotene, phytoene, lycopene, zeaxanthin and lutein) in rice, potato, canola, tomato; folates in rice, corn, tomato and lettuce; vitamin C in corn and lettuce; polyphenolics such as flavonol, isoflavone, resveratrol, chlorogenic acid and other flavonoids in tomato; anthocyanin levels in tomato and potato; iron and zinc in transgenic rice (Matoo et al., 2009). The concept of developing nutritionally functional food requires: (1) the understanding of the mechanisms of prevention and protection; (2) the identification of the biologically active molecules, and (3) the demonstrated efficacy of these molecules with human subjects.

Databases are growing with information on the content of phytonutrients in edible vegetables and fruits as well as with transgenic technology developments for enhancing the nutritional content of vegetable crops *via* engineering of specific metabolic pathways. Metabolic pathway engineering approaches have demonstrated the power of genetic manipulation in enhancing the content of nutrients beneficial for human health in transgenic crops; as well as key genes to inhibit production of allergenic proteins or toxins in crops is highly sought (Matoo et al., 2009).

Therefore, genetic engineering was used to produce β-carotene in rice endosperm. Daffodil (*Narcissus pseudonarcissus*) *psy* gene, which encodes phytoene synthase and produces the first carotenoid phytoene, a key precursor of β-carotene, was expressed in rice under a CaMV 35S (constitutive) or Gt1 (endosperm-specific) promoter (Burkhardt et al., 1997). This study catalyzed efforts to engineer the carotenoid biosynthetic pathway in rice endosperm using daffodil *psy* gene and bacterial (*Erwinia uredevora*) phytoene desaturase *crt1* gene (Ye et al., 2000; Beyer et al., 2002). Transgenic rice seeds were yellow in colour due to the accumulation of carotenoids mainly β-carotene and, to some extent, lutein and zeaxanthin. Several studies have shown that *psy* gene product catalyzes a limiting and regulatory step in carotenoid biosynthesis. The maximum carotenoid accumulation was achieved with maize *psy*, increasing the total carotenoid level to 37 µg/g, some 23-fold higher than a previous report on the Golden Rice (Paine et al., 2005).

Transformation of canola seed with bacterial phytoene synthase (*crtB*) gene using seed-specific napin promoter resulted in orange coloured seed with a 50-fold higher (1000-1500 µg/g FW) total carotenoid level than the wild type (33 µg/g FW) (Shewmaker et al., 1999). Canola seeds coexpressing *crtI* and *crtB* gene accumulated lycopene (29 µg/g FW) and β-carotene (857 µg/g FW) (Ravanello et al., 2003). In a seed-specific manner, bacterial *crtB* gene expression in flaxseeds resulted in 7.8 to18.6-fold increase in the carotenoid levels (Fujisawa et al., 2008).

*crtB* gene was also used in a tuber-specific manner to increase carotenoids, violaxanthin, lutein and β-carotene, in transgenic potato (Ducreux et al., 2005). Downstream from the β-carotene pathway are important photoprotective compounds whose levels have also been successfully manipulated *via* genetic intervention. Using the fruit-specific *pds* promoter to drive the expression of *lcy-β* and *chy-β* genes from Arabidopsis and pepper, respectively, about 100-fold increase in β-cryptoxanthin and zeaxanthin was achieved (Dharamapuri et al., 2002).

Ketocarotenoids are rare in plants but are strong antioxidants. They are chemically synthesized and used as dietary supplements and pigments in aquaculture and nutraceutical industry. Ketocarotenoid biosynthesis pathway was engineered in carrot by introducing an algal *Haematococcus pluvialis* β-carotene ketolase gene under the control of constitutive, ubiquitin or *rolB* promoter (Jayraj et al., 2008). This resulted in 70% conversion of total β-carotene to ketocarotenoid that accumulated to a level of 2,400 μg/g root dry weight. These studies showed that these transgenic carrots are suitable for biopharming ketocarotenoid production for functional food, nutraceutical and aquaculture industries.

Phytonutrients are now recognized as important determinants of human health. This has catalyzed investigations into broader aspects of plant-based nutraceuticals. These include: elucidation of biochemical pathways to identify the rate-limiting steps; engineering metabolic pathways to direct the intermediary metabolism flux towards a particular nutrient (nutraceutical); testing efficacy of either an isolated and purified nutraceutical or a crop engineered with an enhanced nutrient level in animal and human models; testing crops silenced for health-detrimental factors including allergens; comparing bioavailability of an individual nutrient (nutraceutical) fed either as a food supplement or in the form of a fortified food (Matoo et al., 2009). A common goal of such studies is to enable dietary intervention in human health to combat monogenic or polygenic diseases.

Progress in merging agriculture with preventive medicine will depend on intense collaborative research between physicians, nutritionists and plant biologists so that planning strategies are rationally designed and the developments in genetic technology successfully applied for better crop engineering (Matoo et al., 2009).

### 1.3 *Chlamydomonas reinhardtii* as biological farm

Eukaryotic microalgae have recently gained interest as biological farms, because they are attractive alternatives to bacterial, yeast, plant and other cell-based systems currently in use (Pulz and Gross, 2004; Walker et al., 2005; Spolaore et al., 2006; Mayfield et al., 2007). Over the last decades there has been considerable progress in genetic engineering technologies for algae. Biotechnology companies now apply these techniques to alter metabolic pathways and express valuable proteins in different cell compartments. Particularly, the eukaryotic unicellular alga *Chlamydomonas reinhardtii* (Figure 9), sometimes called “green yeast” (Goodenough, 1992; Rochaix, 1995), portraits an important niche in cell biology eukaryotic world and nowadays become a model organism widely used in laboratories thanks to the availability of complete genome sequence, including chloroplast and mitochondrial genomes,

annotated databases for any searching or cloning requirements and successful high-level expression of recombinant proteins in the chloroplast compartment (Mayfield et al., 2007; Manuell et al., 2007). Besides, *C. reinhardtii* belongs to the group of green algae included in the category of organisms with a GRAS status (Generally Regarded As Safe) granted by the FDA. Thus, enhancing food with edible algae like chlamydomonas engineered or not to (over)produce functional ingredients has the potential to become an important factor in food and feed technologies.

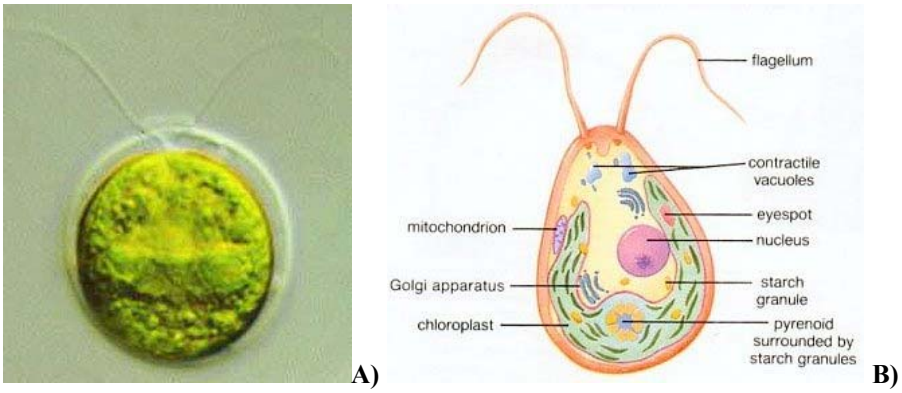



**Figure 9**. **Eukaryotic unicellular alga *Chlamydomonas reinhardtii* A)** A single *C. reinhardtii* cell (from http://www.veda.cz/article.do?articleId=26215). **B)** Schematic diagram of *C. reinhardtii* cell structure (from website: http://universe-review.ca/R10-34-anatomy2.htm#classification).

Moreover, *C. reinhardtii* has been extensively used as an experimental system for studying flagellar motility, function of the basal body, as well as formation and function of chloroplasts, exploiting the powerful of microbiology and genetic engineering techniques (Lefebvre and Silflow, 1999).

Chlamydomonas has interesting biotechnological features including a rapid growth in both liquid and solid media; a sexual cycle, that can be precisely controlled; genetic aspects which enable the generation and characterization of a wide collection of mutants with lesions in structural and metabolic regulator genes and a flexible metabolism being able to growth both phototrophycally and heterotrophycally (Grossman et al., 2003).

Microalgae have long been used as food or food additives. For human nutrition, edible microalgae like *Arthrospira* species ("Spirulina") and *Chlorella* are marketed as tablets, capsules and liquid or added to e.g. noodles, breads and candies to improve their nutritive and health values. Other major commercial strains used as food ingredients are *Dunaliella* and *Aphanizomenon flos-aquae*, whose extracts exhibit health promoting effects (Spolaore et al., 2006). The acceptance of new microalgae strains by the European Commission for Food

Safety has recently been demonstrated with the marine microalgae *Odontella aurita* certified in 2002 as novel food by the French company INNOVALG. While the exploitation of products derived from natural microalgae has a long history, the generation of transgenic microalgae for biotechnological applications has just started to become an attractive system for expressing foreign proteins or other high-value compounds with e.g. antioxidant, colorant, provitamin or therapeutic properties. Nowadays, genetic engineering of algae by introducing and controlling foreign genes has been developed to an extent that allows to exploit these organisms as bioreactors for the production of high-value compounds (Walker et al., 2005).

While agrobacterium-mediated transformation is now the standard method for nuclear transformation, the biolistic approach using accelerated particles (particle gun) turned out to be the preferential procedure for organelle transformation.

The organellar genomes, specifically those of plastids, are particularly attractive for genetic engineering purposes. Not only do chloroplasts naturally produce high amounts of protein, but they are also uniparentally inherited and integrate properly flanked foreign genes *via* homologous recombination into their genome. As opposed to plant nuclear genomes, these properties provide distinct advantages of chloroplast transgenes with respect to bio-safety and epigenetic stability (Maliga, 2004). Genetic engineering of chloroplasts has made particular progress with genes conferring agronomical valuable traits like e.g. resistance to herbicides (Daniell et al., 1998; Lutz et al., 2001), to fungal and bacterial diseases (DeGray et al., 2001) or to insects (Kota et al., 1999; McBride et al., 1995). Chloroplasts have also been employed to overproduce biopharmaceuticals (Daniell et al., 2002; Staub et al., 2000). Chloroplast genetic engineering is currently most advanced in higher plants, particularly in tobacco (Maliga, 2004). While higher plants offer several advantages over expression systems in other organisms, there are distinct drawbacks like e.g. the length of time necessary for their generation or concerns about containment of transgenic plants in the environment even in trans-plastomic lines (Ellstrand, 2001; Ellstrand, 2003; Ruf et al., 2007). The use of microalgae like *C. reinhardtii* provides interesting alternatives: from vector construction to lab-scale culture volumes of the transgenic strain it takes about 6 weeks and cells can be easily contained in photobioreactors (Johanningmeier et al., 2009).

*C. reinhardtii* was the first organism for which stable chloroplast transformation was reported (Boynton et al., 1988). Early selection methods used cloned chloroplast genes to rescue photosynthetic mutants or chloroplast gene constructs that confer resistance to herbicides (Przibilla et al., 1991) or antibiotics (Newman et al., 1991), later on transformant selection

was mainly based on the expression of bacterial markers conferring antibiotic resistances (Goldschmidt-Clermont, 1991; Bateman and Purton, 2000).

Recent progress in engineering *C. reinhardtii* chloroplasts is the result of 20 years research on developing transformation techniques, improving codon usage and finding efficient promoters and un-translated regions (UTRs) for boosting foreign gene expression.

Today several transgenes have been successfully expressed in *C. reinhardtii* chloroplasts. Although most of them are intended to be used as reporters and thus serve basic research purposes, now several proteins for pharmaceutical applications appear in the list. In particular, the expression of a bioactive mammalian protein was achieved, whose level is estimated to be above 5% of total cellular protein (Mayfield et al., 2007). In the near future it appears possible that such transgenic chlamydomonas strains could be ingested orally without the need for extensive purification of the bioactive compound. This perspective is supported by experiments, in which chlamydomonas cells expressing an epitope of a pathogenic bacterium infecting salmonids were fed to trout. An immune response was observed when transgenic cells were added to the fish food (Patent application US020030022359) (Griesbeck et al., 2006).

The use of microalgae as green cell factories assures a significantly faster generation of stable transgenic lines and is often coupled with high yields, lower costs and rapid, controllable growth in simple media. Microalgae are not hosts to major pathogens and recombinant strains can be grown in full containment, excluding the risk of contaminating natural populations. Moreover, methods exists which allow to construct transgenic algae without employing antibiotic resistance genes (Debuchy et al., 1989; Ferris, 1995) thus minimizing any consumer-based concerns regarding the transfer of marker genes from a food product into the cells of the body.

## 1.4 *Chlamydomonas reinhardtii* mutants

### 1.4.1 *C. reinhardtii* NPQ mutants

A set of *C. reinhardtii* mutants carrying single and double mutation within carotenoid biosynthetic genes were, included in this study (kindly provided by Prof Niyogi, University of California, Berkeley, Department of Plant and Microbial Biology, U.S.A.). These mutants (*lor1*, *npq2* and *npq2 lor1*) are impaired in the biosynthetic steps that lead to the synthesis of xanthophylls and α-carotenes derivatives (Figure 10).

In particular, the *lor1* mutant (Niyogi et al., 1997b; Anwaruzzaman et al., 2004) is defective in the lutein accumulation and its derivative loroxanthin (Chunayev et al., 1991); therefore, this mutant lacking in the final product of the α-carotenes biosynthetic branch, while has a normal xanthophyll cycle. Remarkably, this mutant was generated by UV mutagenesis and selected for its phenotypical altered colour (Tellenbach, 1984). It took some years to achieve a more advanced characterization in pigment composition (Chunayev et al., 1991; Niyogi et al., 1997b), and more to discover that a nonsense mutation (a single G/C-to-A/T base pair change) occurred within the third exon of the *lyc-ε* nuclear gene, encoding the chloroplast-localized enzyme lycopene ε-cyclase, LCYE (Anwaruzzaman et al., 2004).

In the *npq2* mutant (Niyogi et al., 1997), the mutation impairs the zeaxanthin epoxidase activity, responsible for the conversion of zeaxanthin to antheraxanthin and violaxanthin. Consequently, the *npq2* mutant accumulates zeaxanthin and lack antheraxanthin, violaxanthin and neoxanthin. This mutant was generated by insertional mutagenesis of the parental strain (CC-425) by plasmid transformation containing the wild-type *ARG7* gene. Because integration of transforming plasmid DNA into the nuclear genome of Chlamydomonas, occurs primarily by non-homologous recombination (Debuchy et al., 1989; Kindle et al., 1989), transformation results in random mutagenesis (Tam and Lefebvre, 1993; Davies et al., 1994) due to genes interruption or deletion owing to plasmid DNA integration. DNA serves as a convenient tag to facilitate eventual isolation of the gene affected in the mutants (Tam and Lefebvre, 1993; Gumpel et al., 1995; Davies et al., 1996). $Arg^+$ transformants were grown photoautotrophically and identified using a digital video-imaging system with high light (1200 μmol photons $sec^{-1}$) (Niyogi et al., 1997) (Figure 11), which excited chlorophyll fluorescence and induced NPQ.

The constitutive presence of zeaxanthin in *npq2* is not sufficient for qE, which still requires a high ΔpH. However, in *C. reinhardtii npq2* the lifetime of Chl fluorescence is lower even in low light in the absence of a high ΔpH (Holub et al., 2000), and induction of qE by illumination with high light is much more rapid in the mutant compared to the wild type (Niyogi et al., 1997). Recently, a series of *npq2* mutants in *C. reinhardtii* has been characterized at a molecular level, and mutations were found in a gene that is homologous to the *zep* genes in *A. thaliana*, tobacco, and rice (Baroli et al., 2003).

The double mutant was obtained as a spontaneous *npq2* mutant from the *lor1* background (Chunayev et al., 1991; Niyogi et al., 1997, 1997b). Thus, this *npq2 lor1* double mutant lacks the lutein and loroxanthin as well as all zeaxanthin-derived xanthophylls (Polle et al., 2001).

All the aforementioned mutants were backcrossed at least four times to the *C. reinhardtii* CC-125 wild type (www.chlamy.org).

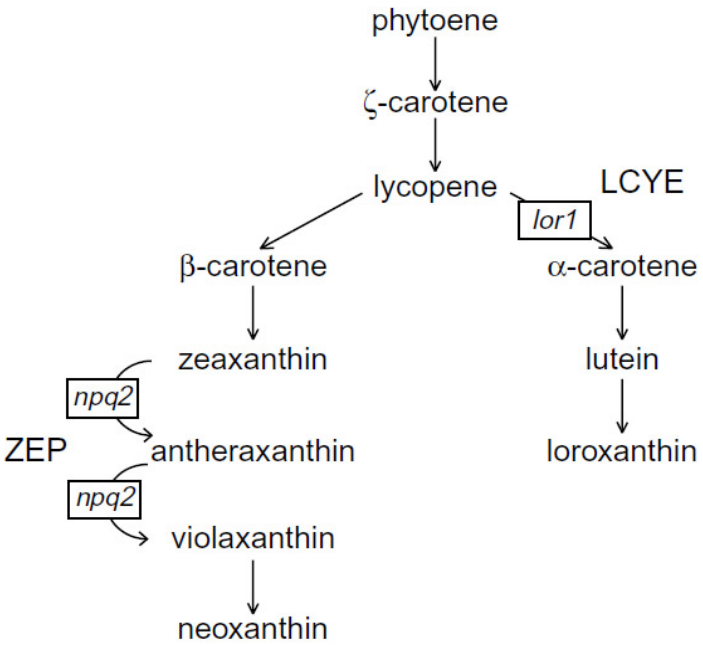



**Figure 10. The xanthophyll biosynthetic pathway in *C.s reinhardtii*.** The reactions catalyzed by ZEP and LCYE are impaired in the *npq2* and *lor1* mutants, respectively(Anwaruzzaman et al., 2004).

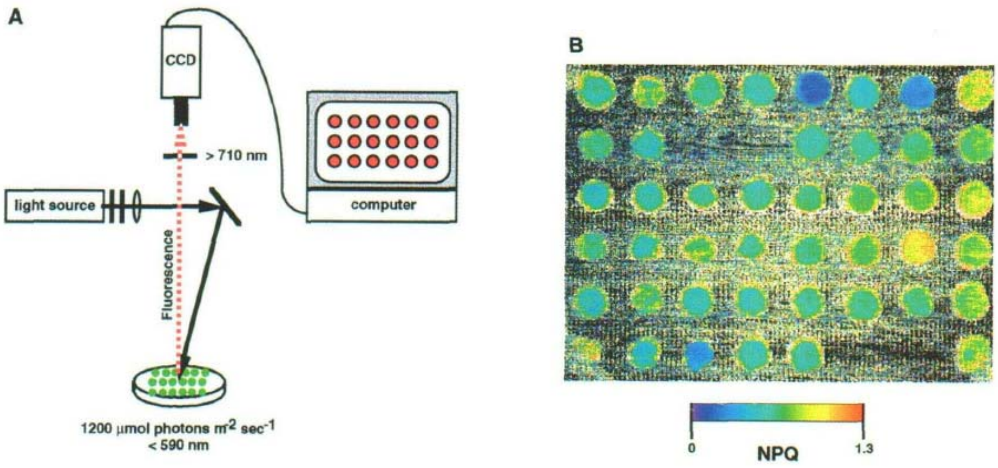



**Figure 11. Screening for *npq* mutants by Digital Video Imaging of Chlorophyll Fluorescence**. **A** Schematic diagram of the video-imaging system. CCD, charge-coupled device. **B** Example of an NPQ image from the screen for mutants. NPQ occurring between 0 and 5 min of illumination with high light was calculated from digitized images of chlorophyll fluorescence. Colonies with a wild-type level of NPQ appear green, whereas mutants with less NPQ appear blue. (Niyogi et al., 1997).

### 1.4.2 *C. reinhardtii* D1 mutants

D1 protein, encoded from *psb*A gene located on the chloroplast genome, is currently one of the most extensively studied thylakoid membrane proteins. Due to its functional homology to the L subunit of the crystallized bacterial reaction centre (Deisenhofer et al., 1985), its structure and membrane arrangement has been worked out in considerable detail (Svensson et al., 1990; Tietjen et al., 1991; Trebst A, 1986).

Specific amino acids and functionally important regions in D1 are recognized to play a role in $Q_B$, herbicide, pigment and metal binding as well as in electron transfer reactions (Vermaas et al., 1991; Oettmeier W, 1992).

During translation the protein is segmentally inserted into the thylakoid membrane (Kim et al., 1991) and translational regulation is mediated by specific proteins interacting with its mRNA at the 5' end (Danon and Mayfield, 1991). At the DNA level, the sequences of more than 30 *psb*A genes have been determined to date (Svensson et al., 1991). Of these, only *C. reinhardtti* (Erickson et al., 1984), *C. smithii* (Palmer et al., 1985), *C. moewusii* (Turmel et al., 1989) and *Euglena gracilis* (Karabin et al., 1984; Keller and Stutz, 1984) *psb*A genes are interrupted by introns. C. *reinhardtii* contains four large group I introns ranging in size from 1.1 to 1.8 kb.

A transformant with no introns in the *psb*A gene was obtained (Johanningmeir and Heiss, 1993) and represented the first example of the removal of a complete set of introns from a chloroplast gene. The newly generated strain is photosynthetically competent and contains no detectable recipient genome copies. The loss of all four introns appears to be phenotypically silent.

A set of *C. reinhardtii* mutants carrying single mutations in the chloroplastic *psb*A gene, were included in this study (kindly provided by Prof. Johanningmeier, Martin-Luther-University Halle-Wittenberg, Institute of Plant Physiology, Germany).

The *psb*A site-specific mutants were generated by the chloroplast biolistic transformation of the IL strain (Johanningmeier et al., 1993), containing the intronless *psb*A gene and 1.9 kb of 3' flanking sequences. The selected mutants are A250R (Ala250→Arg) and S264K (Ser264→Lys), which have functional photosynthetic activity and a resistant phenotype to different classes of herbicides (Przibilla et al., 1991; Johanningmeier et al., 2000). Moreover, some mutants in A250 and S264 have been reported to be resistant to very high light intensities and ionizing radiation (Forster et al., 2001).

## 2. PROJECT AIM

The thesis was supported by the European Project "Nutra-Snack" (E.U.N. FOOD-CT-2005-023044), whose aim was the production of "Ready to eat food for breakfast and sport activity with high content of nutraceutics reducing a disease risk and promoting public health".

This project gave us the opportunity to carry out basic research studies of photosynthesis whose results can find biotechnological applications in agrofood sciences.

The activities were finalised to deeply characterize photosynthetic efficiency and capacity of *Chlamydomonas reinhardtii* strains, mutated in the PSII D1 proteins and impaired in the biosynthetic pathway of carotenoids, hence in non photochemical quenching mechanism (so called NPQ mutants) as well as in relation to their capacity to produce photosynthetic pigments relevant for human health.

For this purposes we decided to work with unicellular biflagellate *Chlamydomonas reinhardtii* algae which can produce photo-protective carotenoid pigments.

Carotenoids are crucial in the human diet, for this reason they are targets for metabolic breeding and/or engineering of various species, in order to improve plant yield and nutritional quality. Microalgae are one of the main sources of natural carotenoids. They can be an excellent model to study the carotenogenesis and are ideal hosts for genetically modified endogenous or exogenous genes that allow the production of carotenoids or higher quantities of the formerly produced ones. In this context we performed expression analyses of biosynthetic carotenogenesis genes, which are necessary and preliminary to any metabolic engineering project, particularly under stress conditions. A combination of high light and high temperature treatment was selected in our studies.

# 3 MATERIAL AND METHODS

## 3.1 Growth conditions and cell count

Cells were grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium, pH 7.2 (Harris, 1989) with stirring at 150 *rpm* at 25°C (Certomat BS-1 incubator, Sartorius) under continuous light at 50 μmol photons/$m^2$/s provided by fluorescent lamps particularly strong at the blue and red ends of the spectrum and thus ideal for promoting photo-biological processes in photosynthetic organisms (OSRAM Fluora). When necessary these media were solidified with 1.5% noble agar (Difco).

The cell growth rate were checked by measuring the cell density and chlorophyll content (Arnon, 1949) at 750 and 652 nm respectively by spectrophotometer Perkin-Elmer Lambda 40 UV/VIS (Perkin-Elmer, Norwakl CT., USA).

Cells were counted by binocular microscope (Leitz Diavert) in a Thoma chamber (0.100 mm Tiefe Depth profondeur, 0.0025$mm^2$) after adding 0.1 ml of Lugol's iodine solution for microscopy (Sigma) to 0.9 ml of *C. reinhardtii* culture of different concentrations.

## 3.2 *Chlamydomonas reinhardtii* strains

### 3.2.1 D1 strains

**IL reference strain**

Sources: CC-4147 FUD7 mt+, from Jacqueline Girard-Bascou.

Comment: The recipient strain for IL was the deletion mutant FuD7 [Johanningmeier U, Heiss S. Construction of a *Chlamydomonas reinhardtii* mutant with an intronless *psb*A gene. Plant Mol Biol. 1993 Apr;22(1):91-9]. From this strain, the Del mutant has been obtained as previously reported [Preiss S, Schrader S, Johanningmeier U. Rapid, ATP-dependent degradation of a truncated D1 protein in the chloroplast. Eur J Biochem. 2001 Aug;268(16):4562-9]. The Del mutant has been used as a recipient strain to generate the mutants by a procedure outlined by Dauvillee et al. [Dauvillee D, Hilbig L, Preiss S, Johanningmeier U. Minimal Extent of Sequence Homology Required for Homologous Recombination at the *psb*A Locus in *Chlamydomonas reinhardtii* Chloroplasts using PCR-generated DNA Fragments. Photosynth Res. 2004;79(2):219-24].

Derived mutants were obtained by site direct mutagenesis: **A250R** (Ala 250 Arg) and **S264K** (Ser 264 Lys) mutants carried single mutations in the chloroplastic *psb*A gene (Johanningmeier et al., 1993), were included in this study (kindly provided by Prof.

Johanningmeier, Martin-Luther-University Halle-Wittenberg, Institute of Plant Physiology, Germany).

### 3.2.2 NPQ strains

**CC-125 wild type mt+ 137c**

Allele nit1-137

Allele nit2-137

Source: R.P. Levine via N.W. Gillham, 1968

Comment: This is the basic "137c" wild type strain originally from G.M. Smith, isolated in 1945 near Amherst MA, and is presumably equivalent to strain 11/32c of the Culture Centre of Algae and Protozoa. This particular strain was brought to Duke by N.W. Gillham in 1968 from Levine's laboratory at Harvard. CC-124 and CC-125 carry the nit1 and nit2 mutations, and cannot grow on nitrate as their sole N source. CC-125 carries the AGG1 (agg1+) allele for phototactic aggregation; see *The Chlamydomonas Sourcebook* [Harris (1989)], p. 215. Contrast CC-124, which has the agg1 allele at this locus. CC-125 is the background strain for many mutations, and is a good control for most purposes.

Derived mutants: ***npq2***, generated by insertional mutagenesis of the parental strain (CC-425) by plasmid transformation containing the wild-type *ARG7* gene (Niyogi et al., 1997a), ***lor1***, generated by UV mutagenesis and selected for its phenotypical altered colour (Tellenbach, 1984), ***npq2 lor1***, obtained as a spontaneous npq2 mutant from the lor1 background (Chunayev et al., 1991; Niyogi et al., 1997a, 1997b).

## 3.3 Antenna size determination

Antenna size determinations were performed by analysing the Chl *a*/cell ratio. 900 µl of Chlamydomonas culture (optical density $OD_{750}$= 0.4) were collected and the cells movement were blocked with 100 µl filtered Lugol's solution (Sigma). Spectra were recorded on an Perkin Elmer Lambda Bio 40 spectrophotometer, and Chl *a* and *b* were calculated using the equation of Lichtenthaler (1987). The cells were counted manually using a Thoma's chamber (0.100 mm Tiefe Depth profondeur, 0.0025$mm^2$). At least three separate repetitions per each strain were done.

## 3.4 Fluorescence measurements

### 3.4.1 Chlorophyll fluorescence

Chlorophyll fluorescence was measured on algae cultures in TAP medium (7 ml final volume with final optical density $OD_{750}$=1) by Fluorescence Monitoring System (FMSII, Hansatech, King's Lynn, Norfolk, UK). The fluorescence emission was measured at room temperature for 2.5 s, the duration of the saturated pulse was 0.7 s. The values for the minimal (*Fo*) and the maximum (*Fm*) fluorescence are automatically calculated by the instrument. Prior to each measurement, the samples were kept in dark for 10 min in order to reach PSII dark-adapted state that is required for the registration of the real *Fo* (corresponding to the fluorescence from open reaction centres). After illumination with saturated white light pulse, the fluorescence signal increases up to its maximum, *Fm*, as a result of reduction of $Q_A$, the primary quinone acceptor of PS II – fluorescence from closed reaction centres. Only the reaction centres which are able to reduce $Q_A$ (*i.e.* photochemically active reaction centres) can contribute to the variable fluorescence yield, *Fv=Fm-Fo*.

### 3.4.2 Oxygen evolution analyses

Photosynthetic activity of the cells was measured by Clark-type oxygen electrode (S1 electrode disk) connected to Chlorolab 2 System and liquid-phase electrode chamber DW2/2 (Hansatech Instruments, King's Lynn, Norfolk, UK). An aliquot of 0.8 ml cell suspension was transferred to the oxygen electrode chamber. All samples contained similar chlorophyll content (about 18 μg/ml), which allowed the comparison of the registered signal between the different samples. To ensure that oxygen evolution was not limited by the carbon source available to the cells, 200 μl of a 50 mM sodium bicarbonate solution (pH 7.4) was added prior to the oxygen evolution measurements (Melis et al., 1999). The samples were illuminated with increasing light intensities (10, 30, 50, 80, 100, 200,300, 350, 400, 500, 600 and 0 μmol photons/$m^2$/s), provided by 11 red LED Light Source LHII/2R centred on 650nm, (Hansatech Instruments, Pentney, England), under continuous stirring (70 *rpm*) and at a temperature of 25°C. The rate of oxygen evolution under each light intensity was recorded continuously for a period of 2 min. The $O_2$ evolution rate on chlorophyll bases were plotted as a function of the light intensity.

## 3.5 Quantitative and qualitative analyses of photosynthetic pigments

### 3.5.1 Pigment standards and HPLC system

All standards (carotenoids, xanthophylls and chlorophylls) were bought with the highest purity. All other chemicals and solvents were HPLC or ACS grade (Carlo Erba, Milan, Italy).
All the pigment standards were prepared at the suitable concentration for the necessary calibration of HPLC curves, by diluting the stock standards solution under dim (or green safe) light and at 4°C. Nitrogen gas was flushed into vials to avoid pigments degradation and isomerization. All the vials were stored at –20°C and in the dark. The HPLC instruments (Gilson, Milan, Italy) encompass a gradient system (two 306 pump, 811c dynamic mixer, 805 manometric module, 155 UV/Vis detector) and a Rheodyne mod 7125 injection valve (20 µl loop). The HPLC system and the chromatographic data were controlled by a 506 interface (Gilson) from the Unipoint Gilson software. The HPLC column was an YMC C30 column (Waters, USA), 5 µ, 250 x 4,6 mm id, plus a C18 (1 cm) guard column and in-line filter (0,22 µm). Both the HPLC and the guard columns were thermostated by the "Croco-Cil" HPLC column heater (Sainte-Foy-la-Grand, France).
Standards were diluted/dissolved in degassed 100% iced acetone in amber vials at stock concentrations under Nitrogen flux (GC grade), and dim lights. Stocks were kept frozen at –20°C. Working standard solutions were diluted with the HPLC solvent mixture methanol-acetonitrile-water (84:14:2, v/v/v) (solvent A) to avoid solvent interference during the run. We chose the solvent A, after different tests, because it showed the best peaks resolution. The HPLC calibration curve of each standard was analyzed at least three times. No by-products in the standards were detected by HPLC analyses for a period of at least three months.

### 3.5.2 Carotenoids and chlorophylls extraction

*C. reinhardtii* cultures were centrifuge 15 min at 4°C at 3500 *rpm* and the growth medium was discarded. Immediately, samples were frozen at -80°C. Subsequently, samples were thaw on ice and lyophilized at -50°C under vacuum overnight. The exact dry weight of each sample was measured to normalize all samples on mg dry weight basis. A small amount of HPLC grade water (200 µl) was added to allow the next extraction steps. Re-hydrated samples were supplemented with some crystals of Na-carbonate to avoid a drop of the pH due to the lyses in the sample; immediately after, samples were re-suspended in 100% cold acetone. After vortexing and centrifuging (15 min at 4°C at 3500 *rpm*), samples were re-suspended in 1 ml of 100% cold acetone or until the pellet was bleached. Before HPLC injection, solutions were

filtered through a 0.45 μm PVDF filter and dried under $N_2$ bubbling. Hence, extracts were diluted with 400 μl of a mixture composed by solvent A and placed in amber vials. Samples were stored at -20°C until analysis.

### 3.5.3 Pigments analyses

Each 20 μl injection was performed by the "full loop" technique and each sample was analyzed almost three times (3 independent runs). When necessary for confirmation, standards were added to the sample extract for co-chromatography.

The most suitable mobile phase system comprised a mixture of solvent A and 100% methylene chloride (solvent B) with the following gradient condition [time (%A, %B)]: [10 (100, 0)], in 10 min [10 (95, 5)], in 15 min [10 (80, 20)], in 10 min [5 (50, 50)], in 5 min [10 (100, 0)]. The flow rate was 1.0 ml/min. Pigment detector was set at 440 nm (carotenoids and xanthophylls) and 640 nm (chlorophylls). The column temperature was maintained at 21°C.

The HPLC column was an YMC C30 column (Waters, USA), 5 μ, 250 x 4,6 mm id, plus a C18 (1 cm) guard column and in-line filter (0,22 μm). Both the HPLC and the guard columns were thermostated by the "Croco-Cil" HPLC column heater (Sainte-Foy-la-Grand, France).

## 3.6 Chlamydomonas nucleic acids purification

### 3.6.1 Genomic DNA purification

Total DNA was extracted from 5 ml of *C. reinhardtii* cultures (IL strain) at a density of approximately $2\times10^6$ cells/ml. Cells were spun down by centrifugation at 3500 × *rpm* for 10 min and resuspended in 1.5 ml of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) supplemented with 400 μg/ml of RNase A and incubated at 37°C for 30 minutes. SDS and proteinase K were added to a final concentration of 0.5% and 100 μg/ml, respectively, and incubated 1 hour at 37°C. After that, NaCl was added to a final concentration of 800 mM. Samples were extracted with 1 volume of phenol/chloroform/isoamilic alcohol (25:24:1) equilibrated at pH 8.3. Total DNA was precipitated with ethanol and resuspended in DNAse-free water.

### 3.6.2 RNA extraction and cDNA synthesis

Chlamydomonas culture at $OD_{750}$ 0.4 (corresponding to: IL $22\times10^5$ cells; A250R $55\times10^5$ cells; S264K $64\times10^5$ cells; CC-125 $73\times10^6$ cells and *npq2 lor1* $69\times10^6$ cells) were harvested by centrifugation (at 4°C for 10 min at 4500 *rpm*) and the cell pellet was suspended in 2 ml of DEPC-treated extraction buffer (50 mM Tris-HCl (pH 8), 0.3 N NaCl, 5 mM EDTA and 2%

w/v of sodium dodecyl sulphate). Proteinase K solution (40 µg/ml final concentration) was added and the samples were left on room temperature for 15 min at 250 *rpm*. The resulting cell lysate was extracted twice with phenol:chloroform (1:1) (phenol chloroform equilibrated to pH 4.3, Sigma), and RNA was precipitated overnight in 2 volumes of 100% EtOH. The precipitate was washed once with 70% EtOH and finally dissolved in 40 µl of RNase-free water. The quality of RNA was checked loading 500 ng onto a 1.2% formaldehyde-agarose gel, and, after quantification by NanoDrop™ Spectrophotometers (Thermo Scientific, Wilmington, USA), DNase treatment was performed (RQ1 DNase 1U/µl, Promega) incubating 30 minutes at 37°C. The samples were extracted with phenol:chloroform (1:1) (phenol chloroform equilibrated to pH 4.3. Sigma), and RNA was precipitated overnight adding LiCl (4M final concentration) in $H_2O$ DEPC and 2.5 volumes of 100% EtOH at -80°C. The precipitate was washed once with 70% EtOH and finally dissolved in 20 µl of RNase-free water.

The reverse transcription reaction was performed to convert 1 µg of total RNA into first-strand cDNA by SuperScript ™ III (First strand Synthesis System for RT-PCR, Invitrogen), combining 50 µM Oligo(dT) and 10 mM deoxynucleotide triphosphates (dNTPs) with RNA template and heating at 65°C 5 min and then rapidly cooling the reaction to avoid the generation of RNA secondary structures. Then the Superscript™ III enzyme was added (200 U per reaction) and incubated 50 minutes at 50ºC. The reaction was terminated incubating 5 min at 85°C. Finally the samples were treated with RNase H (2 U per reaction) 30 min at 37°C and cDNA synthesis reaction checked by PCR using rDNA 18S gene primers.

### 3.7 Chlamydomonas nucleic acids analyses

#### 3.7.1 DNA electrophoresis on agarose gel

The DNA fragments, from PCR amplifications, were analyzed by electrophoresis on horizontal 1% agarose gel and 1 µg/ml ethidium bromide in buffer 1×TAE (0.04 M Tris-acetate, 1 mM EDTA). The estimation of the DNA fragments size was performed by a marker of molecular weight (Hyper Ladder I, Bioline). During the analysis DNA samples were subjected to a potential difference of 75 volts, for different periods depending on the size of analyzed DNA and the degree of separation required. DNA labelled with ethidium bromide was visualized on a transilluminator with UV light (312 nm).

### 3.7.2 RNA electrophoresis on agarose gel in denaturant conditions

All solutions were treated with 0.1% dietilpirocarbonato (DEPC) before use. Total RNA was fractionated on agarose gel 1.2%. The agarose was dissolved in a buffer solution containing: 20 mM 3-morfolinpropansolfonico acid (MOPS) pH 8.0, 5 mM sodium acetate, 1 mM EDTA, formaldehyde to a final concentration of 6% was subsequently added . RNA samples containing 50% formamide, 6% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 0.1% Bromophenol blue, 0.1% xylene cianolo, and 0.5 μg / ml ethidium bromide, were denatured at 65°C for 5 min and loaded on gel. Electrophoresis was conducted at 60 mA for the first 10 minutes and at 50 mA for 3 hours in buffer containing: 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA. The fractionated RNAs were visualized by illumination with UV light.

## 3.8 Polymerase chain reaction (PCR)

### 3.8.1 Oligonucleotides design

Specific oligonucleotides were designed to amplify gene encoding carotenoid and plastoquinone biosynthetic enzymes, utilizing genomic information available at the Chlamydomonas Center portal (www.chlamy.org) (Table 1). Oligonucleotides were used in PCR experiments.

### 3.8.2 PCR standard conditions

For the amplification, 500 ng of cDNA was added to a 50 μl reaction mixture containing 1.25 U of *DreamTaq* DNA polymerase (Fermentas), 5 μl of 10× *Taq* buffer with $MgCl_2$, 1.25 μl of dNTPs (0.25 mM each) and 1 μM of each primer (0.05 μM for 18S, D1 and D2 primers). PCRs were run on PTC-0150 Mini Cycler system (MJ Research) as follows: initial denaturation (5 min at 94°C), 30 cycles (30 s at 94°C; 30 s at 61°C; 1 min at 72°C) and final elongation (10 min at 72°C). A positive (*C. reinhardtii* genomic DNA) and negative controls were always included in all PCR reactions. A control reaction after RNA extractions was systematically run to rule out the presence of amplification from genomic DNA.

20 μl of amplification product were loaded onto a 1% agarose gel. Exceptions are the *psb*A and *psb*D genes of which 10 μl were loaded.

| Gene | Primer sequence (5'→3') | Amplified fragment size (cDNA bp) | *C. reinhardtii loci* | *A. thaliana* orthologous |
|---|---|---|---|---|
| *psy* | TGGATGAGCTGGACAAGT<br>GTCCGTGAAGTATTGCCG | 140 | AY604702 | At5g17230 |
| *chy-β* | CAACCTGCCGTACATGAA<br>CCCAGAAACATGCCGAA | 90 | XM_001698646 | n.d. |
| *pds* | ACCATGACTGAGCTGGAG<br>CTTGTACTTGCGGATCTTGG | 78 | XM_001690807 | At4g14210 |
| *zds* | CCGACACCTACTTCAGCT<br>TTCGTCCAGGGCATGTA | 127 | XM_001700734 | At3g04870 |
| *lyc-ε* | CAAGTTCCTGCGCTATGA<br>GCCTGAAATCCATGAAGGTC | 128 | XM_001696477 | At5g57030 |
| *lyc-β* | TGACGCTGTTCTGGAAGA<br>CTCCTTGAGCGACATTGT | 140 | AY860818 | At5g57030 |
| *zep* | AGGACAGCCGCATTTTG<br>TCAACCCACTTGCTCCA | 112 | XM_001701649 | At5g67030 |
| *hst1* | ACCAGCCTCTACACCTTT<br>TGTACACGCCGAAGTTGA | 136 | XM_001695289 | n.d. |
| *vdr1* | CCTTCTACTTGTCGGTATTGG<br>TTCTCATCGCAGTCCACA | 73 | XM_001694990 | n.d. |
| *vte3* | TGAGAACGTGACGCTGAT<br>ACGTAGCGGTCAAAGGT | 138 | XM_001692660 | At3g63410 |
| *mpbq-mt* | CACCCCTACTTCATCTCCAT<br>GTGTTCTTGTTCCAGTCCTC | 95 | XM_001692671 | n.d. |
| *psb*A | ACACTTGGGCAGACATCA<br>AGGGAAGTTGTGAGCGTT | 77 | NC_005353 | AtCg00020 |
| *psb*D | TGCACCGTGTAAAGCAAC<br>GCTGTTTCTACACCTGCTAAC | 129 | FJ436959 | AtCg00270 |
| *rack1* | TGCAAGTACACCATTGGC<br>CCAGACCTTGACCATCTTGT | 123 | XM_001698013 | n.d. |

**Table 1.** Specific primers, amplified fragments size and primers concentration used for gene PCR analysis. Orthologous genes from *Arabidopsis thaliana* are also reported.

### 3.8.3 RNA-Retrotranscription (RT) and Real-Time PCR (RT-PCR)

RNA samples were reverse transcribed and amplified using the SYBR Green PCR Master Mix and MuLV Reverse Transcriptase Reagents (Applied Biosystems, Foster city, USA), following the one-step RT-PCR protocol recommended by the manufacturer. About 80 ng of mRNA in 25 µl SYBR Green PCR Master Mix 1X, with 0.25 U/ml MultiScribe Reverse Transcriptase (Applied Biosystems, Foster city, USA), 200 nM forward and reverse primers

(Table 1) were subjected to the following thermal profile: one step at 42°C for 30 min, one step at 95°C for 10 min, 40 cycles with a denaturation step at 95°C for 15 s and an annealing/extension step at 60°C for 15 s. PCRs were performed in the Applied Biosystems 7900HT Real Time System with standard 96-Well Block Module, using Frosted Subskirted optical tubes and Seal Film (Applied Biosystems, Foster city, USA). The reactions were subjected to a heat dissociation protocol present in the 7900HT System software for melting curve analysis and detection of non-specific amplifications: at the end of the final PCR cycle, the amplification products were heat denatured over a 35°C temperature gradient at 0.03°C/s from 60 to 95°C. A negative control without template was run with every assay to assess the overall specificity. For each gene, a common threshold setting applied to each of the two biological replicates determined the threshold cycle (TC). Relative abundance of each gene was determined by the $2^{DDCt}$ method (Livak and Schmittgen, 2001). RACK1 gene (receptor of activated protein kinase C1) (Schloss, 1990) was used as the endogenous control for calculation of relative abundance. Each assay included triplicate reactions.

Primers design and their optimization in regard to primer dimer, self-priming formation and primer melting temperature was done with a melting temperature of 59°–60° and a product size between 90 and 150 bp were designed using Primer Quest (Integrated DNA Technologies, Coralville, IA, http://www.idtdna.com). The complete set of primers used are listed in Table 1.

**3.9 Elicitation experiments: high light and high temperature treatments**

The *C. reinhardtii* D1 mutants, A250R and S264K, and the reference strain, IL,, in early exponential growth phase, were exposed to a combination of heat (37° C) and light stresses (1000 μmol photons/$m^2$/s) under continuous stirring (150 *rpm*). Samples were collected simultaneously from the control and form the treated cell cultures at 0, 15, 30 and 90 min. The following parameters were controlled at each of the indicated times: cell densities, total chlorophyll content, chlorophyll fluorescence, HPLC profile of photosynthetic pigments by and expression level of genes involved in carotenoids and plastoquinone pathways.

Subsequently, in an attempt to increase the total content of antioxidant compounds, the light intensity and the temperature were increased up to 2000 μmol photons/$m^2$/s and 50°C in an experiment with the IL, CC-125 and *npq2 lor1* for 30 minutes.

# 4. RESULTS AND DISCUSSION

## 4.1 Selection of the strains and experimental set up

Cell culture systems represent a potential source of valuable compounds, flavors, fragrances, and colorants, which cannot be produced by chemical synthesis. Hence, we decided to exploit the biflagellate unicellular green alga *Chlamydomonas reinhardtii* to produce functional photosynthetic pigments, like xanthophylls and carotenes. The eukaryotic cells of *C. reinhardtii* have metabolic characteristics typical of prokaryotic organisms. These cells can grow both autotrophycally and heterotrophically using acetate as a carbon source. Large quantities of cells can be produced quickly with relatively simple and inexpensive media. Thus, *C. reinhardtii* is a promising "biological factory" for the production of valuable compounds.

Chlamydomonas owns three genetic systems located in the nucleus, in the chloroplast and in the mitochondria, all sequenced and available at the JGI Chlamydomonas Genome Portal (www.chlamy.org). The newly available information concerning the sequence and organization of the *C. reinhardtii* genomes combined with the many types of physiological, genetic and molecular manipulations, make *C. reinhardtii* the organism of election for these studies (Merchant et al., 2007).

A set of *C. reinhardtii* mutants carrying single and double mutations in the carotenoid biosynthetic genes were included in this study (kindly provided by Prof Niyogi, University of California, Berkeley, Department of Plant and Microbial Biology, U.S.A.). These mutants are impaired in the biosynthetic steps that lead to the synthesis of xanthophylls and α-carotenes derivatives. In particular, we chose the *lor1* mutant (Niyogi et al., 1997b; Anwaruzzaman et al., 2004) whose mutation blocks the accumulation of lutein and its derivative loroxanthin (Chunayev et al., 1991); therefore, this mutant is defective in the final product of the α-carotene biosynthetic branch, while has a normal xanthophyll cycle. The second mutant is the *npq2* (Niyogi et al., 1997a); the *npq2* mutation impairs the zeaxanthin epoxidase activity, which is responsible for the conversion of zeaxanthin to antheraxanthin and violaxanthin. Consequently, the *npq2* mutant accumulates zeaxanthin and lacks zeaxanthin derived. The double mutant, *npq2 lor1* (Polle et al., 2001), was also included in our study. The *npq2 lor1* double mutant lacks lutein and loroxanthin as well as all zeaxanthin-derived xanthophylls. All the above mentioned mutants were backcrossed at least four times to the *C. reinhardtii* CC-125 wild type (www.chlamy.org). Therefore, the CC-125 strain was included in all experiments and used as the control strain.

A second set of *C. reinhardtii* mutants carrying single mutations in the chloroplastic *psb*A gene encoding the D1 protein was also included in this study (kindly provided by Prof. Johanningmeier, Martin-Luther-University Halle-Wittenberg, Institute of Plant Physiology, Germany). In particular, we worked with mutants carrying amino acid substitutions in residues A250 and S264, which constitute part of the $Q_B$ binding pocket in PSII and whose modifications can dramatically alter the electron transport chain and photochemistry. Since the D1 mutants were produced on the bases of the *psb*A intronless IL strain, it was included in all experiments and used as a control reference strain. The selected D1 mutants, A250R (Ala250→Arg) and S264K (Ser264→Lys), have functional photosynthetic activity and are resistant to different classes of herbicides (Przibilla et al., 1991; Johanningmeier et al., 2000). Moreover, some mutants in position A250 and S264 have been reported to be resistant to very high light intensities and ionizing radiation (Forster et al., 2001; Giardi personal communication). It is well known that high light acclimatation induces the production of photoprotective pigments (Havaux and Niyogi, 1999). We hypothesized a positive correlation between herbicide resistance and high light tolerance in order to evidence an increased accumulation of anti-oxidant compounds analysing the potential high light tolerance of D1 A250R and S264K mutants.

At the outset, besides the strains selection, we identified the most suitable analyses and techniques to achieve the project aim. Thus, once the best growth conditions for physiological growth were set up, our research was focused on identification and optimization of the techniques necessary to obtain a complete characterization of the selected strains such as High Performance Liquid Chromatography (HPLC), chlorophyll fluorescence and oxygen evolution.

**Pigment profiling of *C. reinhardtii* and difficulties in their HPLC analyses**

Several factors make pigment determination and quantification in plants quite difficult. Thus, in spite of the advanced and high performance analytical instrumentations of HPLC technique, mismatches in the absolute quantification of the pigments content could be encountered during multi-day analyses. Errors come mainly from sample preparation and chromatographic detection such as losses or partial extraction of pigment, isomerization and oxidation. Other sources of errors are incompatibility of the injection solvents and the mobile phase, erroneous peak identity assignment (more than 600 pigments are known), impurity and standards instability or lack of certified standards, highly overlapping peaks, low recovery from the HPLC column, technical errors, etc. To avoid or minimize these errors, we made a

lot of efforts in the optimization of the pigment extraction conditions, preparations of standards and samples for the HPLC runs, and their analyses. Moreover, most of our preliminary work was aimed at optimizing both carotenoids and xanthophyll pigments profile analyses.

Analyses of carotenoids and xanthophylls are very complex, because of their heterogeneity (a very big number of compounds with minimal structural differences) and the presence of *cis/trans* isomers. The characteristic conjugate double bonds of carotenoids cause the main problem in sample manipulation; their particular instability especially towards light, heat, oxygen and acidic or alkaline conditions can change their composition in the sample. For these reasons, several and strict precautions are necessary to handle these compounds such as operation under dim light, evaporation under a nitrogen stream, storage of the samples in an amber vials and in dark, under nitrogen flux and at about –20°C.

Structurally, carotenoids are polyisoprenoid compounds. All carotenoids are produced by variations of the base of C40 skeleton. Two main groups can be distinguished: hydrocarbon carotenoids, named carotenes which contain only of C and H, and the oxidized carotenoids, named xanthophylls that present some $O_2$-substituent groups such as hydroxyl, keto and epoxy groups. The carotenoids spectrophotometric and functional characteristics result from the numerous conjugated double bonds as well as of the presence of lineal or cyclic groups, like cyclohexane and cyclopentane, located at the both ends of the molecule. The combination of these end-groups with oxygen containing functional groups and changes in the hydrogenation level are responsible for each peculiar structure of carotenoids, thereby complicating HPLC analyses. Moreover, multi-peak formation and peak deformation have been detected during the HPLC analyses. The reasons have been associated with the solubility of the carotenoids in the HPLC eluents, the nature of the injection solvents and the interaction between the pigment and the metal surface.

Clearly, the column characteristics (C-18 *vs* C-30, monomeric *vs* polymeric) and the overall C column loading (its residual polarity) have a very strong effect on the carotenoids separation and on the *cis/trans* isomer separation.

To date, a plethora of HPLC methods can be found in the current literature but most of them regard the use of the C-18 columns. Only recently, C-30 (polymeric or monomeric) columns are commercially available. In this type of column, the length of the C-30 alkyl chain is similar to that of the carotenoid molecules and, hence, more extensive interactions might occur, thereby improving the obtained results. The C-30 column should be preferred when it is mandatory to separate *cis/trans* isomers (since they can differ in biological activity) or the

pattern of carotenoid degradation or when very complex samples have to be analyzed. Keeping in mind all mentioned above, we developed a protocol for the extraction of carotenoids from *C. reinhardtii* samples (see material and methods).

**Fluorescence measurements**

The capacity of the photosynthetic organisms to carry out photochemistry, is limited and depends upon a range of factors including stress caused by the environmental conditions. Absorbed light energy in excess must be effectively dissipated by non-photochemical processes (npq). Such processes include the emission of heat and re-emission of small but significant amounts of the absorbed radiation as longer wavelength red/far-red light energy. This re-emission of light is called chlorophyll fluorescence. The photochemical and non-photochemical processes operate in direct competition and any change in energy utilization by one process produces a complementary change in the others. Although chlorophyll fluorescence emission from whole photosynthetic systems is too weak to be captured by naked eye, it can be detected with a proper instrumentation. Peak chlorophyll fluorescence occurs in the red region of the spectrum (around 700 nm) and extends into the infra-red region to around 800 nm.

The fluorescence method is widely used to characterize the functionality of PSII in photosynthetic organisms. The maximum quantum yield of the PSII photochemical reaction ($F_v/F_m$) is a reliable parameter which describes the efficiency of the photosynthetic apparatus under different environmental conditions.

Thus, the chlorophyll fluorescence technique was used as a rapid and reliable non-invasive probe of PSII photochemistry, measuring algae in TAP medium by Fluorescence Monitoring System (FMSII). For the purpose of these experiments, a new script was compiled: the intensity of the modulation beam was set on its maximum and 100% gain was applied to the fluorescence signal. Moreover, the fluorescence signal of *C. reinhardtii* cultures with different cell concentration was measured in order to improve the ratio signal/noise and to obtain reliable measurements. As a result of the screening tests that were performed, a *C. reinhardtii* cultures concentrated up to $OD_{750}$ = 0.9 were used. The chlorophyll fluorescence emission was measured at room temperature for a period of 2.5 s, the duration of the saturated pulse was 0.7 s.

**Oxygen evolution**

The measurement of photosynthetic oxygen evolution by polarographic electrode is one of the most sensitive and reliable method to determine efficiency of photochemistry. In photosynthetic process, the absorbed light energy is used to drive the reduction of carbon dioxide to carbohydrate, using $H_2O$ as a donor of electrons and resulting in release of oxygen. Hence, if a photosynthetic organism is enclosed in a chamber and supplied with a source of carbon dioxide (for example bicarbonate) and illuminated, oxygen will be evolved and could be detected polarographically by a Clark-type electrode (Clark, 1956).

Hence, the registration of the oxygen evolution rate provides a direct measurement of the efficiency of the photosynthetic reaction and is an important source of physiological data; the method was employed in our study resulting in the development of a new protocol. The crucial parameters as the electrode calibration (between the oxygen levels of air saturated water and zero oxygen), stirring (70 *rpm*) and temperature (25°C) were set up. Moreover, in order to obtain a significant signal of the evolved oxygen which will allow us to compare the photosynthetic rates of the different samples, the Chl concentration in the oxygen electrode chamber was fixed at 18μg/ml.

### 4.2 Characterization of *C. reinhardtii* strains under physiological conditions

The characterisation of the physiological state of chlamydomonas was performed utilizing a set of methods and parameters which gave a general estimation of the main vital processes in the selected strains such as growth rate, total chlorophyll content, PSII efficiency, $O_2$ evolution capacity as well as photosynthetic pigments profile.

#### 4.2.1 Growth curve

The characterisation of the growth rate is an important point in the selection process of the chlamydomonas strains in order to be utilized as a biological farm for the production of valuable compounds.

The photoheterotrophic growth rate of D1 and NPQ strains was characterized on cultures grown on TAP nutrition medium under contentious illumination (50 $\mu mol/m^2/s^1$ of white light) and stirring (150 *rmp*) at 24 °C temperature and determined spectrophotometrically by measuring the culture optical density ($OD_{750}$) and chlorophyll content for a period of 168 h (Figure 1).

In the initial growth phase (the first 88 h, $OD \leq 1$) the D1 mutants and the control strain IL showed no differences in $OD_{750}$, which assumed similar growth rate (Figure 1A). In the

exponential phase (1.0 ≤ OD ≤ 2.0) the IL strain grew faster than the A250R and S264K mutants, but for all D1 strains the exponential phase was observed in the range of 96 and 112 hours of growth (Figure 1A). During the plateau (2.0 ≤ OD ≤ 3.0) both D1 mutants reached higher $OD_{750}$ values compared to IL (after the first 136 h of growth), which indicates the ability of the mutants to maintain higher number of cells during the late culture growth phase. During all developmental phases, IL strain accumulated much bigger amount of chlorophyll per ml in comparison with the two D1 mutants and in particular with S264K; this difference was particularly noticeable in the plateau phase of the growth curves of the chlorophylls accumulation (Figure 1B). Taking in account that the culture density is proportional to the cell number per ml, these results suggested that the mutants accumulate less chlorophyll per cell in comparison to the reference strain IL.

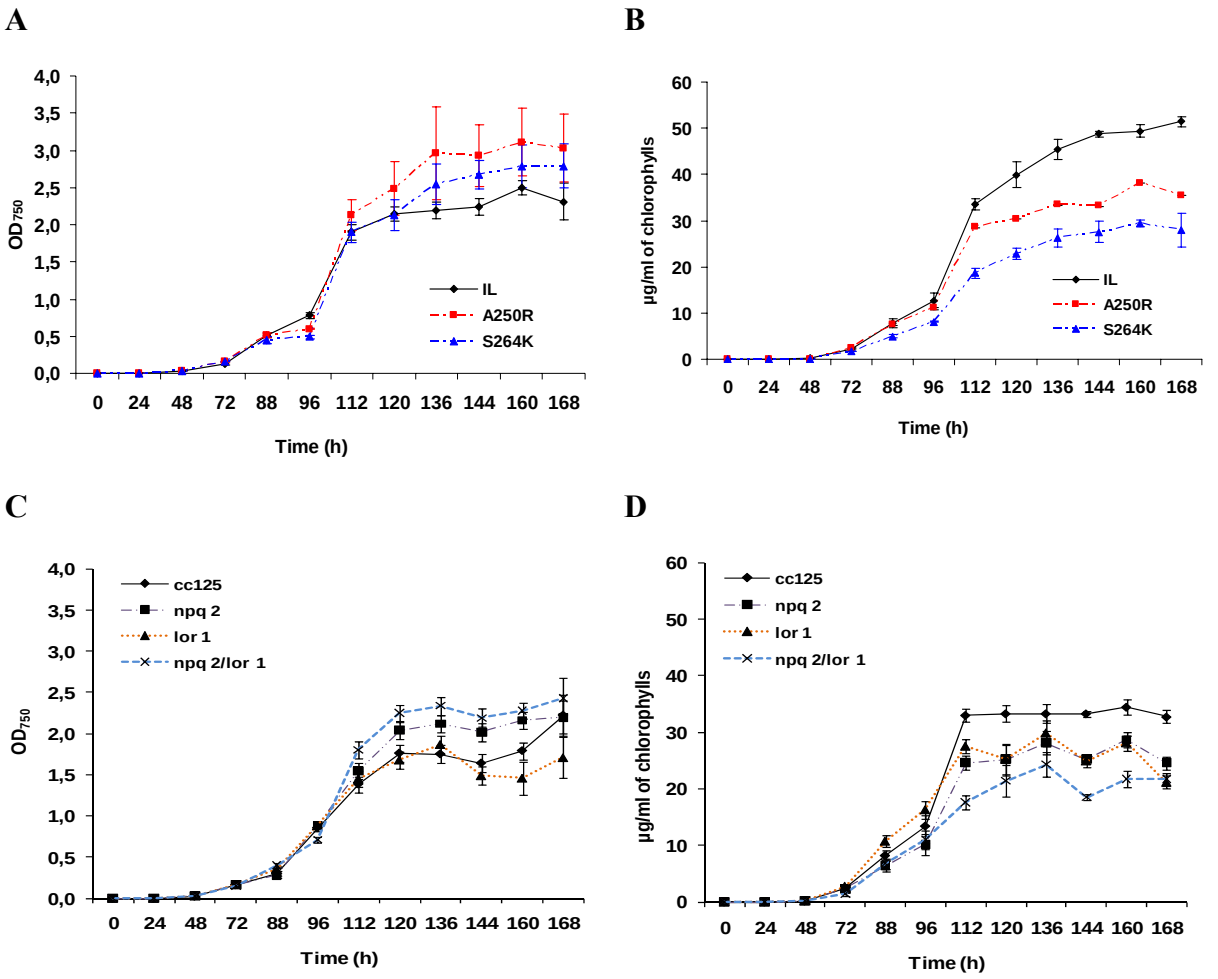



**Figure 1. Growth curves and chlorophyll content of C. *reinhardtii* D1 and NPQ strains.** Optical density ($OD_{750}$) and total chlorophyll content (µg/ml) of *C. reinhardtii* D1 strains (A and B) and NPQ strains (C and D). At least three separate repetitions were done per each strain. Each point represents the average of three experiments ± SD.

No significant differences were noted in the initial and the exponential growth phases ($0 \leq OD \leq 2.0$) among the NPQ strains (Figure 1C). The exponential growth occurred in the first 96 and 112 hours of the culture development as it was observed for the D1 strains and estimated by both optical density and chlorophyll accumulation curves (Figure 1C). During the curve plateau ($2.0 \leq OD \leq 2.5$), as observed in the case of the D1 strains, NPQ mutants reached higher $OD_{750}$ values compared to CC-125 after 120 hours of growth (Figure 1C).

During all developmental phases, CC-125 strain produced more total chlorophyll per ml in comparison with the NPQ mutants and in particular with *npq2 lor1*; this difference was particularly evident in plateau phase of the curves of the chlorophyll accumulation (Figure 1D).

Comparison of D1 and NPQ strains growth parameters showed a similar behaviour between wild type strains and the corresponding mutants; in fact both IL and CC-125 showed lower optical density values and higher total chlorophyll content in comparison with their mutants during the growth. Besides, in the exponential phase of chlorophyll curve relative to IL strain, a bigger amount of total chlorophyll content compared to CC-125 was observed (Figure 1B and D).

### 4.2.2 Chlorophyll fluorescence

The fluorescence measurements were included to evaluate the quantum efficiency of PSII photochemical reaction and to characterise the rate constants of the electron transfer through the PSII core complex.

The polyphasic rise of the chlorophyll a fluorescence exhibited upon illumination of a dark-adapted photosynthetic sample reflects mainly the photoinduced reduction of the PSII electron carriers and transition of the reaction centres into a closed state. According to the present dogmatics, the fluorescence intensity under saturating light rises from the initial dark-adapted level (*O, $F_0$*), where all $Q_A$ are oxidised, to the maximal level (*P, $F_m$*), corresponding to a state where all reaction centres are closed. The fluorescence induction curve, measured with a microsecond time resolution has two intermediate steps – *J* (around 2 ms) and *I* (around 30 ms) – reflecting the transient concentration of reduced $Q_A$ (reviewed recently by Lazar, 2006). The *V(t)* fluorescence transients, ($V(t)=(F_i-F_0)/(F_m-F_0)$), that reveals only the dynamic accumulation of the reduced $Q_A$ in the analyzed D1 mutants are presented in Figure 2. The lower down rate of the $Q_A^-$ reoxidation, reflected by the increase in the level of the fluorescence transients, clearly demonstrated the effect of the aminoacid substitution in the D1 protein on the electron transport rate through the PSII. The efficiency of the electron

transfer between the primary ($Q_A$) and the secondary ($Q_B$) quinone acceptor in PSII, presented in Figure 2 calculated as $1\text{-}V_J$ according to Strasser et al., (2000), decreased remarkably in the order IL>A250R>S264K.

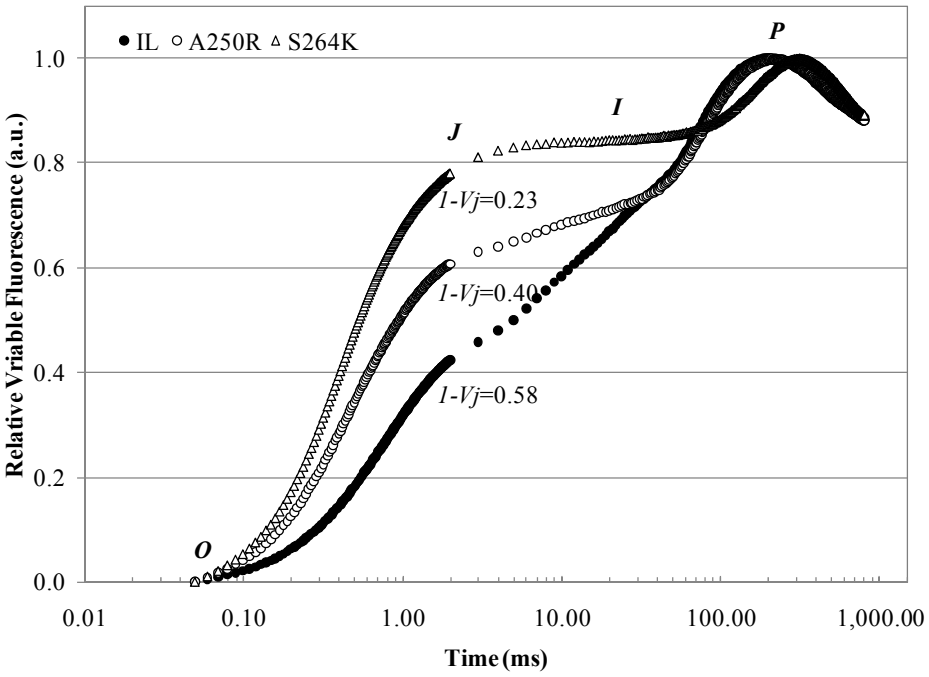



**Figure 2. Curves of the relative variable fluorescence (*V(t)*) of the D1 strains**. The *V(t)* curves were calculated as $V(t)=(F_t\text{-}F_0)/(F_m\text{-}F_0)$. The O, J, I and P characteristic points are indicated.

On the bases of the rate constants of the competing energy dissipation processes in the photosynthetic organisms, it was shown that the ratio of variable ($F_v=F_m\text{-}F_0$) to the maximal fluorescence (Fv/Fm) is directly proportional to the maximal quantum efficiency of PSII photochemistry (Kitajama and Butler, 1975). This ratio is extremely conservative (Björkman and Demmig, 1987) and is the most widely used fluorescence parameter for general estimation of the photosynthetic reactions. In the presented study, the Fv/Fm ratio was used as a fast and reliable screening parameter for the evaluation of the strength of the applied stress factors.

Table 1 shows a summary of the single mutations presented in the studied chlamydomonas strains with the corresponding Fv/Fm values. Under physiological conditions, the reference strains, IL and CC-125, showed similar Fv/Fm values (0.810 and 0.813 respectively) which were notably higher in comparison with the respective mutants.

| Strain | Mutation | Fv/Fm |
|---|---|---|
| **Mutants in D1 protein** | | |
| **IL** | *psb*A gene without introns, (Johanningmeier et al., 1993) | 0.810 ± 0.005 |
| **A250R** | Ala250Arg in the binding niche for $Q_B$<br>Resistance against different class of herbicides (Johanningmeier et al., 2000) | 0.784 ± 0.003 |
| **S264K** | Ser264Lys in the binding niche for $Q_B$<br>Resistance against different class of herbicides (kindly provided by Prof. Johanningmeier) | 0.658 ± 0.014 |
| **Mutants in carotenoids biosynthetic genes** | | |
| **CC-125** | Wild type (www.chlamy.org) | 0.813 ± 0.004 |
| ***npq2*** | Impaired in the conversion of zeaxanthin in antheraxanthin (Niyogi et al., 1997b) | 0.731 ± 0.008 |
| ***lor1*** | Unable to make α carotene, lutein and loraxanthin because it is defective in ε-cyclase activity (Niyogi et al., 1997) | 0.779 ± 0.010 |
| ***npq2 lor1*** | Accumulation of zeaxanthin and β carotene (Polle et al., 2001) | 0.715 ± 0.008 |

**Table 1. Structural features and biochemical characterization of D1 and NPQ strains.** Chlorophyll *a* fluorescence analyses indicated that S264K and npq2 lor1 have lower photosynthetic efficiency compared to relative control strains. Each point represents the average of three measurements ± SD.

These data led to the conclusion that the D1 aminoacid substitution of Arg in position 250 and Ser in position 264, which are directly involved in the formation of the $Q_B$ binding niche, straightforward alter the photosynthetic electron transfer rate and reduce the PSII efficiency.
Instead, in the case of the NPQ mutants, the link between the alterations in the carotenoid biosynthetic genes and the lower PSII performance was not so linear. In that case the analyses of the *OJIP* fluorescence transients did not reveal any difference in the electron transport rate through PSII core complex (data not shown). Most probably, the modified photosynthetic pigment profile, resulted in changes in the in the LHC organization and composition, could influence the light absorption and excitation transmission processes in the PSII complex, and therefore the electron transport in the thylakoid membrane.

### 4.2.3 Oxygen evolution capacity

To compare the efficiency of light energy conversion in the cells of the reference strains and mutants, the rate of $O_2$ evolution was measured and plotted as a function of the light intensity. The light dependency curves of the $O_2$ evolution consists of two phases (Polle et al., 2001; Walker, 1990). The first phase represents the linear increase in the $O_2$ evolution with the increase in the intensity of the incident light. This phase is used to calculate the initial slope of the curve, which represents the quantum yield or in other words the efficiency of the light energy utilization in the process of $O_2$ evolution. During the second phase the slope of the curve gradually decreases and a plateau is reached. The rate of $O_2$ evolution under saturated light intensity is considered a measure for the capacity of the photosynthetic reaction.

In D1 strains, the wild type IL showed a higher capacity for $O_2$ evolution than the mutants as it is evident from the light dependency curves presented in Figure 3. In spite of the big difference in the capacity for $O_2$ evolution between the reference strain (78 ± 2 μmol $O_2$/mg Chl/h) and the mutants, A250R (56 ± 2 μmol $O_2$/mg Chl/h) and particularly S264K (33 ± 2 μmol $O_2$/mg Chl/h), the photosynthetic reactions in all the strains were saturated at the same light intensity (around 300 μmol/$m^2$/s). According to Melis et al., 1999 it means that all the strains have similar effective antenna size.

The results obtained for $O_2$ evolution capacity are in agreement with the PSII performance, evaluated by Fv/Fm ratio (Table 1). The alteration in the electron transport rate through the PSII complex in the D1 mutants resulted also in reduction in the $O_2$ evolution rate.

The quantum yield of the photosynthetic reactions is cross function of the light absorption capacity and electron transport efficiency and can be estimated by the initial slope. Most likely the impaired electron transfer in the mutants (especially in the case of S264K) led to the lower efficiency of the light energy utilization in comparison to the IL strain (Table 2). We should take in account also the possible contribute to the low photon use efficiency of the quantity of absorbed light, which is proportional to the size of antenna. In addition to the lowest initial slope found for the S264K, this mutant showed the highest light compensation point (76 μmol photon/$m^2$/s) among the D1 strains (Table 2). The latter is calculated as the light intensity at which photosynthetic $O_2$ evolution reaches equilibrium with the respiratory $O_2$ uptake, and depends on the antenna size and the rate of dark respiration (Walker, 1990). In Table 2 the dark respiration values corresponding to the oxygen consumption in absence of light are also reported. These data indicated a higher oxygen consumption in the S264K mutant (27 μmol $O_2$/mg Chl/h) compared to wild type IL and A250R mutant (around 20 μmol

$O_2$/mg Chl/h). For this reason we assumed that the S264K mutant could own a more rapid metabolism activity in the dark than the reference strain, IL, and A250R mutant.

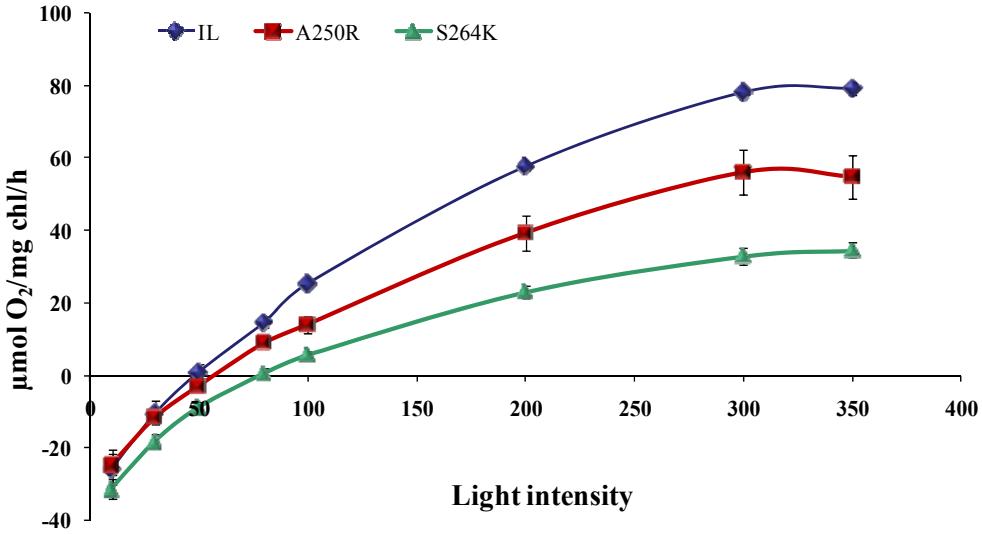



**Figure 3. Light dependency curves of the oxygen evolution in D1 strains.** The evolved oxygen in D1 strains is reported as a function of different light intensities (from 0 to 350 µmol photon/$m^2$/s) provided by red LED Light Source, under stirring and at a temperature of 25 C°. At least three separate repetitions were done per each strain. Each point represents the average of three experiments ± SE.

| | IL | A250R | S264K |
|---|---|---|---|
| **Slope (arbitrary unit)** | 0.5 ± 0.1 | 0.4 ± 0.0 | 0.3 ± 0.0 |
| **Light compensation point (µmol photon/$m^2$/s)** | 48 ± 5 | 59 ± 3 | 76 ± 4 |
| **Dark respiration (µmol $O_2$/mg Chl/h)** | 21 ± 1 | 20 ± 1 | 27 ± 2 |

**Table 2. Photosynthetic activity of the D1 strains cells.** The analyses by Clark type polarographic oxygen electrode indicated that the wild type IL has a higher gas exchanges capacity and photosynthetic efficiency compared to the relative mutants, A250R and S264K. The values are means (n=3) ± SE.

The results lead to the conclusion that the reference strain, IL, has the highest photosynthetic performance among the selected D1 mutants. Generally the oxygen evolution is accepted as an estimation of the rate of photosynthesis, so it should be proportional to the production of carbohydrates and components necessary to the growth. The higher performance could give some advantages in the stress resistance of the organism, even if it is not necessarily related to

the secondary production of metabolites, since the two metabolisms are not always going in the same direction.

Concerning the NPQ mutants, it has been demonstrated (Polle et al., 2001) that the photosynthetic efficiency (the value of the initial slope) in CC-125 and in the double mutant *npq2 lor1* is similar 0.14 and 0.13 relative value), and the photosynthetic capacity of the mutant (59 μmol $O_2$/mg Chl/s) is bigger compared to the wild type CC-125 (36.5 μmol $O_2$/mg Chl/s). This behaviour is opposite compared to that observed in the D1 strains and it could be due to the replacement of lutein, loroxanthin and violaxanthin by zeaxanthin, and the consequent smaller antenna size, as it occurred in the *npq2 lor1* mutant. Indeed, the reduction of light harvesting complex dimension in *npq2 lor1*, demands higher light intensity to reach the saturation level of the photosynthetic reactions.

### 4.2.4 PSII antenna size

Chlorophyll antenna size is not fixed and can vary substantially depending on a wide range of factors (Mitra and Melis, 2008 and the references there in). As a response to the environment, the low light intensity during growth promotes a larger Chl antenna size and *vice versa* the high irradiance induces the assembly of a smaller Chl antenna size. Such adjustment is a long-term compensation reaction in response to the incident irradiance intensity. This regulatory mechanism is highly conservative in nature and functions in all photosynthetic organisms. The size of the antenna could be also genetically predetermined, because of alteration in the pigments composition in the light harvesting complex (LHC).

The IL, A250R, S264K, CC-125 and *npq2 lor1* strains were further characterized measuring the PSII antenna size, according to LaRoche et al. (1991). The analysis was carried out determining the Chl*a*/cell ratio as reported in Material and methods and are presented in Figure 4. The obtained results indicated that the reference strains, IL and CC-125, had highest values for this parameter, 0.0086 and 0.0054 pg Chl*a* per cell respectively and most likely biggest antenna size than the mutants. As it was reported in the literature (Niyogi et al., 1997; Polle et al., 2001) the double NPQ (*npq2 lor1*) and its parent *lor1* mutants of *C. reinhardtti* have a truncated PSII antenna size as a result of the altered photosynthetic composition of pigments. The lack of xanthophylls, i.e. antheraxanthin, violaxanthin, lutein and loroxanthin, in this mutants is overcome by their partially replacement with zeaxanthin in the pigment-protein complexes of LHCII but it results in the reduction of the PSII antenna size.

Instead, the reduced antenna size in D1 mutants is not related directly to modification in the carotenoids composition presented in their antenna. We could hypothesize that the slow down

electron transport rate through the PSII core complex in the D1 mutants could results in a decrease of the effective light absorption cross section. This is very likely a defence response in order to adjust the light absorption capacity to the ability to utilized the absorbed energy in the photochemical reaction.

In conformity with the data presented for the Chl*a* per cell, the mutants showed slightly higher Chl *a*/*b* ratio than the respective control strain (not more than 16% higher). This parameter varied in a significant way from 2,5 in the reference strains up to 2,9 in the mutants. In general, the differences in the ratio of the two main light-harvesting pigments in the photosynthetic apparatus are attributed to differences in the intensity of the incident growth light (Melis et al., 1999; LaRoche et al., 1990) –an higher growth light intensity, that induces the assembly of a smaller Chl antenna size, results in an increase of the Chl *a*/*b* ratio (more than 50%, but strongly depends on the species and growth conditions). In addition, the truncated PSII antenna size of the NPQ mutants (Polle et al., 2001; Niyogi et al., 1997) was associated with higher Chl *a*/*b* value in comparison with the wild type.

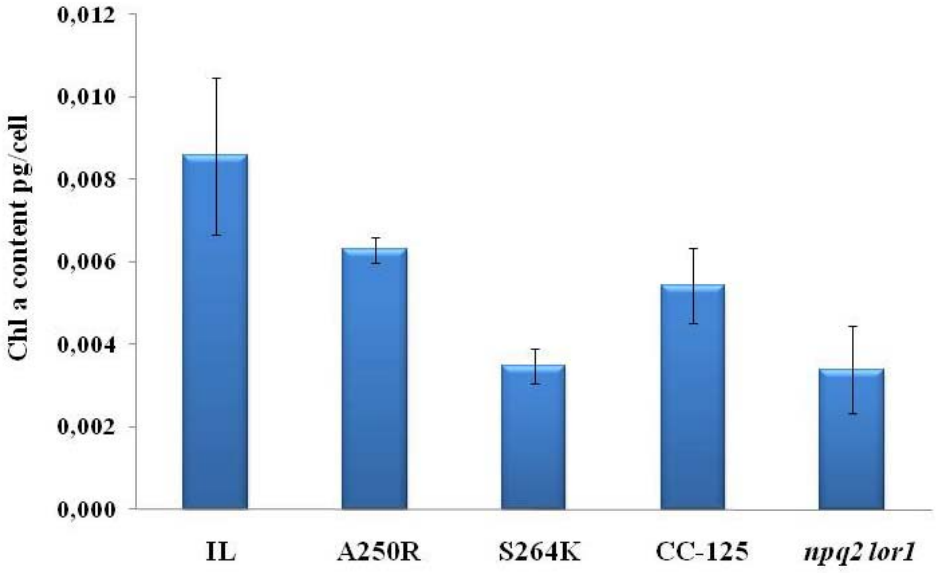



**Figure 4. Chl*a* content per cell in *C. reinhardtii* strains.** The average values of three independent experiments are shown. The ratio Chl*a*/cell was calculated, using the average value for the chlorophyll content and the cell number in each independent experiment. For each independent experiment three internal repetitions were done.

Taking in to account the relatively small differences that we found in the pigment content and the ratio of Chl*a* per cell between the mutants and the control strains, we could hypothesize that also under our conditions a reduction of the effective mutant's PSII antenna size occurred. Our results have to be discussed in the context of the low intensity under which the

strains were grown (50 µmol/$m^2$/s in comparison to 200 µmol/$m^2$/s in the experiments of Polle et al., 2001 and Niyogi et al., 1997). It is very likely that the extent of the PSII antenna size reduction is a function of the incident growth light intensity.

These results made us think that the D1 mutants physiologically behave like high light adapted organisms with smaller PSII antenna size, as it is known for the NPQ mutants. A truncated Chl antenna most probably would inevitably compromise the ability of the strain to compete and survive in the wild conditions. However, under controlled laboratory or in a mass culture bioreactor it would help to diminish the over-absorption and wasteful dissipation of excitation energy by individual cells or the photoinhibition of photosynthesis (Mitra and Melis, 2008). Some advantages concerning the mutants were hypothesized under high light elicitation conditions, since the reduced antenna size could mean a lower "elicitation sensing" due to the lower photons captured.

### 4.2.5 Accumulation of photosynthetic antioxidant pigments

The photosynthetic pigments profile of each organism competent for photoautotrophic growth is a unique characteristic. In the same time, it can vary substantially on the developmental, genetic, physiological and even environmental conditions. The alteration in the quantity and composition of the photosynthetic pigments as a reaction to the changes in the above mention conditions represents an important part of the adaptation and acclimation potential of any photosynthetic organism (Walters, 2005). In this context, the HPLC analyses of the photosynthetic pigments of the selected chlamydomonas strains were important characterisation parameter for their further utilization as a biological farm.

The characterization of the physiological profile of the photosynthetic pigments of the selected strains was performed on algae cultures in the early exponential growth phase. The resulting carotenoids profiles of the analyzed D1 and NPQ strains are presented in Figure 5 and Figure 6, respectively.

The reference strains differed from the corresponding mutant in respect to the total xanthophylls (violaxanthin, anteraxanthin, zeaxanthin and lutein) content. In the case of the D1 strains, the slightly higher content of xanthophylls in IL, compared to the A250R and S264K mutants, was mainly due to violaxanthin. The opposite trend was observed in NPQ strains – the total content of xanthophylls was higher in the mutants in comparison with wild type CC-125. The zeaxanthin was the most abundant xanthophylls in the NPQ mutants and its content increases in the following order *lor1>npq2>npq2 lor1* (Figure 6). The accumulation of zeaxanthin in the double mutant *npq2 lor1* have been also reported by Pole et al., (2001)

and it was described as one of its main characteristics. As a result of the corresponding mutations in *npq2* and *npq2 lor1*, the violaxanthin and lutein were present only as traces (similar data were reported also by Jahns et al., 2000, Polle et al., 2001). In *lor 1* all β-carotene and no α-carotene derivatives were found (as lutein in accordance to Niyogi et al., 1997).

The level of the β-carotene in the D1 mutants was higher than in the reference strain. Also the NPQ mutants accumulate much more β-carotene than the wild type and its content increases in the following order *lor1*>*npq2*>*npq2 lor1*. In all chlamydomonas strains the detected levels of α-carotene were lower than those of *β*-carotene, which is involved in crucial role as the assembly of PSII core complexes and function as scavenger of reactive oxygen species, and as potential electron donors to $P680^+$ (Grossman et al., 2004 and references therein).

As it is evident from Figure 5 and 6, the comparison of HPLC profiles of all strains, showed a notably higher secondary content of pigments in NPQ compared to the D1 strains in the order *npq2 lor1*>*npq2*>*lor1*>CC-125>IL>A250R>S264K.

In conclusion, these results suggest that the best producers of antioxidant photosynthetic pigments among the D1 strains was the wild type IL, and the *npq2 lor1* mutant among the NPQ strains.

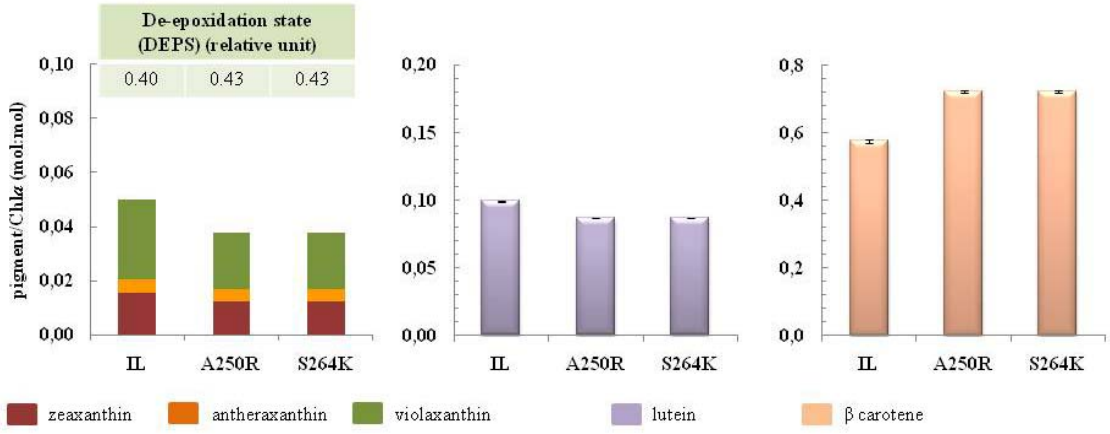



**Figure 5. Photosynthetic pigments accumulation measured by HPLC system in D1 strains.** Xanthophylls (zeaxanthin, anteraxanthin, violaxanthin and lutein) and β-carotene contents related to the chlorophyll *a* in *C. reinhardtii* IL, A250R and S264K strains under physiological conditions. The de-epoxidation state ratio (zeaxanthin+antheraxanthin/zeaxanthin+antheraxanthin+violaxanthin) was also reported. At least three separate runs in HPLC system were done per each strain. The values represent the average ± SD.

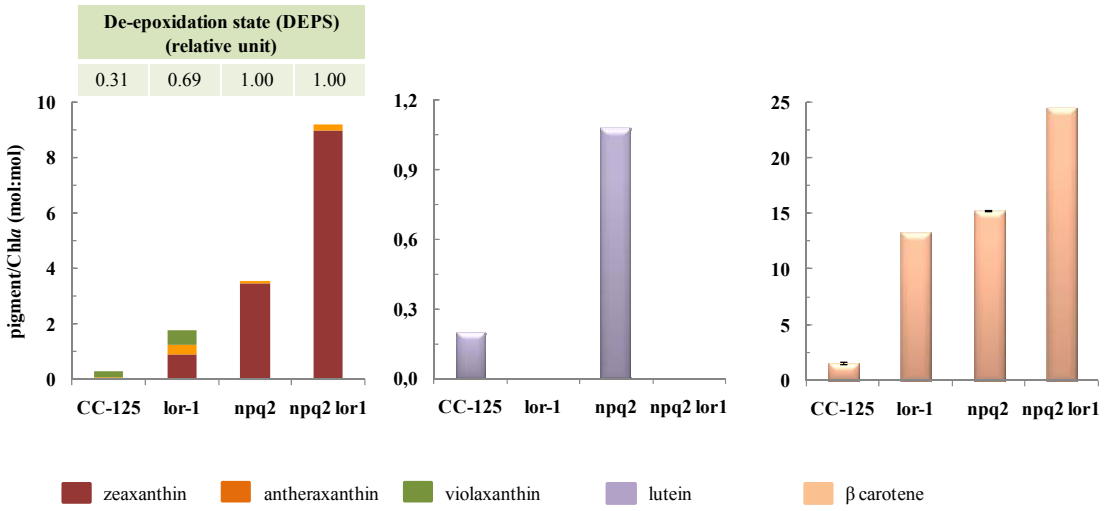



**Figure 6. Photosynthetic pigments accumulation measured by HPLC system in NPQ strains.** Xanthophylls (zeaxanthin, anteraxanthin, violaxanthin and lutein) and β-carotene contents related to the chlorophyll *a* in *C. reinhardtii* CC-125, *npq2*, *lor1* and *npq2 lor1* strains under physiological conditions. The de-epoxidation state ratio (zeaxanthin+antheraxanthin/zeaxanthin+antheraxanthin+violaxanthin) was also reported. At least three separate runs in HPLC system were done per each strain. The values represent the average ± SD.

### 4.3 Elicitation strategies to improve antioxidant photosynthetic pigment accumulation

As mentioned above, one of the major obstacles for the production of plant secondary metabolites by plant cell culture technology is the low yield of metabolites. Since the major role of these chemical compounds is to protect the plants from biotic and abiotic stress (Ferrari, in press), we exploited a set of biological, physical and chemical elicitation strategies to enhance their production.

To this purpose, different chlamydomonas strains were exposed to a wide range of elicitation factors (Table 3), including exposure to UV-C light, a combination of high light/high temperature and treatments with oligogalatturondes (OGs), and sodium nitroprusside (SNP). After the elicitation, the pigment quantification was performed by HPLC technique.

This thesis will describe results obtained after treatment with HL/HT.

#### 4.3.1 Superimposition of high light and high temperature on D1 strains

In order to induce an over-production of pigments with antioxidant activity, *C. reinhardtii* cultures in the early exponential growth phase of IL, A250R and S264K strains, were treated simultaneously with high-light (1000 µmol photons/$m^2$/s) and high temperature (37°C) for a period of 90 min, further indicated as HL/HT treatment. Samples were harvested at 0, 15, 30 and 90 min after the begging of the HL/HT exposure and cell density, total chlorophyll

content, PSII maximum quantum yield (Fv/Fm), pigment composition and expression levels of some genes involved in carotenes and plastoquinone synthesis, D1 and D2 proteins biosynthesis, were analyzed. Figure 7 indicates the enzymes, for which the gene expression levels were analysed and the corresponding biosynthetic steps in which they are involved.

| Elicitation strategies | | Experimental set up |
|---|---|---|
| **UV-C** | It is detrimental to the PSII apparatus leading to impaired electron transport capacity and damage to the protein subunits. | Germicidal lamp; Peak at 254 nm<br>Temperature: 25° C<br>Times: 0, 30, 90, 180 min<br>Strains: IL, A250R and S264K |
| **OGs** | Fragments with a degree of polymerization between 10 and 15 elicit a variety of defence responses including accumulation ROS. | Light intensity: 50 µmol $m^{-2}s^{-1}$<br>Temperature: 25° C<br>Times: 0, 3, 8, 24, 48 hours<br>[OG]: 800 µg/ml (4h dig.)<br>Strains: cw15 |
| **SNP** | SNP releases nitric oxide that functions as a bioactive molecule for adaptive responses of plant fitness to environmental constraints. | Light intensity: 50 µmol $m^{-2}s^{-1}$<br>Temperature: 25° C<br>Times: 0, 2, 4, 6, 24, 48 hours<br>[SNP]: 1.5 mM<br>Strains: IL |
| **HL/HT** | These conditions stimulate production of antioxidant photosynthetic compounds reducing harmful radical effects. | Light intensity: 1000 µmol $m^{-2}s^{-1}$<br>Temperature: 37° C<br>Times: 0,15, 30, 90 min<br>Strains: IL, A250R and S264K |
| | | Light intensity: 2000 µmol $m^{-2}s^{-1}$<br>Temperature: 50° C<br>Times: 0, 30 min<br>Strains: IL, CC-125 and npq2/lor1 |

**Table 3. Screening of *C. reinhardtii* strains and elicitation strategies to select hyper-producing carotenoids organisms.** OGs – oligogalacturonides, SNP – sodium nitroprusside, HL/HT – high light/high temperature.

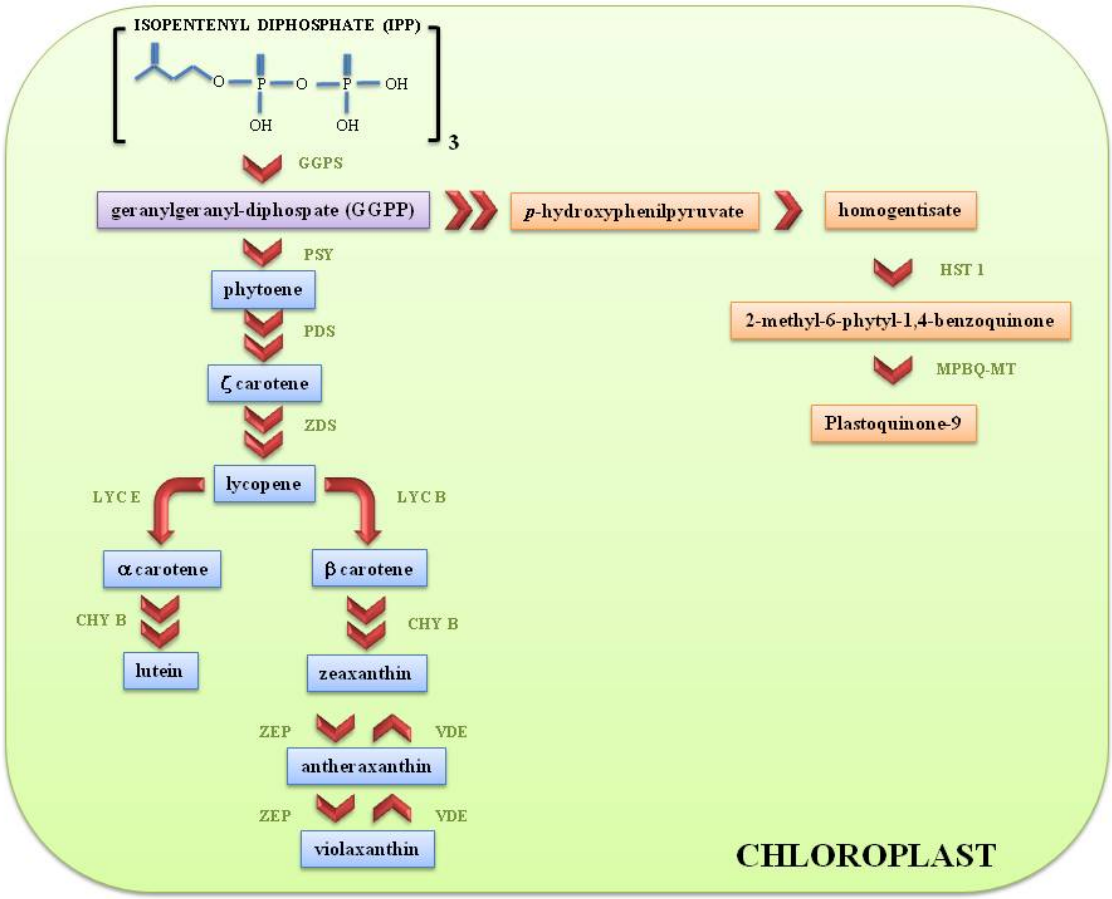



**Figure 7. The main steps in the carotenoid and plastoquinone biosynthetic pathways in *C.reinhardtii* chloroplast.** The fundamental compounds involved in the carotenoids (in blue) and the plastoquinone (in orange) biosynthesis, starting from an isopentenyl diphospate (IPP), are presented. The main enzymes involved in the catalytic steps are reported in green.

### 4.3.2 HL/HT effects on the chlorophyll content and PSII photochemistry in D1 strains

The cultures optical density and total chlorophyll content, were measured spectrophotometrically in each of the indicated times during the HL/HT treatment. No changes in these parameters in both IL and D1 mutants as a result of the treatment were observed. The average value of the $OD_{750}$ for all the strains during the treatment were 0.4±0.02. The variation in the total chlorophyll content during the HL/HT exposure was negligible, as it is evident from the following average values for IL (4.70±0.03 µg/ml), for A250R (4.54±0.06 µg/ml) and for S264K (4.34±0.07 µg/ml). The above reported values were calculated as a mean from the values of the chlorophyll content in the control and in the HL/HT exposed cell cultures. The stability of this parameter during the treatment, measured spectrophotometrically, was confirmed also by the HPLC analyses. Moreover, in the Chl*a*/*b* ratio no changes induced by the exposure to HL/HT for up to 90 min were observed.

The reactions of the photosynthetic electron transport are among the most sensitive physiological processes in respect to changes in the light and temperature conditions. The high light and temperature stress could induce damage of the D1 protein, loss of the oxygen evolving activity, inhibition of electron transfer between the primary and secondary plastoquinone of PSII, dissociation of the peripheral antenna complex of PSII from its core complex (Bukhov and Mohanty, 1999; Cushman and Bohnert, 2000). It was shown that PSII is damaged under severely high temperature stress, while the high-light-induced stimulation of reactive oxygen species production could result directly in damage of the D1 protein (Yamane et al., 1998; Cushman and Bohnert, 2000). As it could be expected, the illumination with irradiance 20 times higher than the growth light intensity in combination with high temperature led to strong reduction in the photosynthetically active PSII reaction centres and considerably decreased the maximum quantum yield of PSII photochemistry (Table 4). The Fv/Fm was reduced in a similar manner in both, mutants and reference strain. Such significant decline in Fv/Fm ratio could be a result of severe damage to the reaction centre of PSII and considerable increase in the proportion of the absorbed energy dissipated through non-photochemical pathways.

We could hypothesize that the strength and/or the duration of the selected elicitation were not strong enough to provoke severe damage in the photosynthetic apparatus, often reported to appear as bleaching of the photosynthetic pigments (Bumann and Oesterhelt; 1995; Minagawa et al., 1996; Fischer et al., 2006). On the other hand, the selected conditions effectively altered the photosynthetic electron transport reactions, changing the actual redox-state of the carriers in the electron transport chain and as it could be expected activating different protective mechanisms to dissipate the light energy in excess. The former have been extensively studied during the last few decades and the literature is abundant of information about the role of the state transition (LHCII phosphorylation), the ΔpH-dependent quenching (including zeaxanthin), the photoinhibition, the rapid D1 turnover and ect. in the protection of the integrity and the function of the photosynthetic apparatus (Aro et al. 1993; Wollman, 2001; Baroli et al., 2003; Szabò et al., 2005; Yamamoto et al., 2008).

### 4.3.3 Antioxidant photosynthetic pigment accumulation during HL/HT treatment on D1 strains

The pigment profile of the strains was determined by HPLC at the indicated times during the treatment in both control and HL/HT exposed cultures. Since the D1 strains had different amount of pigments even under physiological conditions, the content of secondary pigments

was normalized to the quantity of Chl *a* (Figure 8 and 9). This approach allowed the comparison of the relative pigment accumulation between the strains.

| Fv/Fm under 1000 μmol photons/$m^2$/sec and 37°C | | | | | | |
|---|---|---|---|---|---|---|
| | IL | | A250R | | S264K | |
| Time (min) | CTRL | HL/HT | CTRL | HL/HT | CTRL | HL/HT |
| 0 | 0.789 ± 0.001 | 0.789 ± 0.001 | 0.751 ± 0.009 | 0.751 ± 0.009 | 0.705 ± 0.002 | 0.705 ± 0.002 |
| 15 | 0.792 ± 0.002 | 0.659 ± 0.011 | 0.757 ± 0.006 | 0.647 ± 0.024 | 0.715 ± 0.004 | 0.619 ± 0.003 |
| 30 | 0.799 ± 0.002 | 0.639 ± 0.015 | 0.756 ± 0.007 | 0.511 ± 0.029 | 0.699 ± 0.004 | 0.591 ± 0.002 |
| 90 | 0.788 ± 0.003 | 0.459 ± 0.000 | 0.749 ± 0.012 | 0.480 ± 0.032 | 0.706 ± 0.005 | 0.464± 0.007 |

**Table 4. Chlorophyll fluorescence analyses during HL/HT treatment on *C. reinhardtii* cultures.** Reduction of the maximum quantum yield of PSII photochemical reaction (Fv/Fm) in D1 strains, measured during the HL/HT experiment (1000 μmol photons/$m^2$/s and 37°C) . The values represent the average (n=3) ± SD.

The elicitation gradually led to accumulation of the all pigments involved in the xanthophyll cycle (violaxanthin, antheraxanthin, zeaxanthin). The increase was higher in the referent strain, less evident in the A250R mutant and in contrast no stimulation of the xanthophylls cycle was detected in the S264K mutant. The relative content of zeaxanthin undergo the biggest changes (Figure 8). Usually in the photosynthetic organisms the zeaxanthin is absent or present only in small amount under normal light conditions. In our case, at the end of the elicitation its amount increased about 7 times in the IL strain and about 5 times in the A250R mutant. The role of the xanthophylls cycle, which results in accumulation of zeaxanthin, in the ΔpH-dependent quenching of the excess light is a well known phenomenon. It was shown that zeaxanthin has a crucial importance under high irradiance conditions since it is responsible for the direct quenching of the chlorophylls exited singlet state and involved in the conformational change of antenna proteins (reviewed by Szabò et al., 2005). The activation of the xanthophyll cycle towards zeaxanthin formation could be estimated by the calculation of the relative amount of the de-epoxidated xanthophylls (Z+A) over the total xanthophylls content (V+A+Z) that are involved in the cycle. This is the so called de-epoxidation state (DEPS=(Z + A)/(V + A + Z)), also presented in Figure 8. A notable increase in DEPS was found only in the reference strain, IL, in agreement with the higher level of zeaxanthin accumulation. In the IL strain, the elicitation also led to the increase of the relative amount of β-carotene and lutein (Figure 9). Both pigments are also involved in the process of

excess light energy dissipation as heat in the LHCII. In addition β-carotene is an active radical scavenger and an important substrate for zeaxanthin synthesis (Grossman et al., 2004). The use of the β-carotene in the zeaxanthin synthesis could be a reason for the observed recline in its content at 90 min, in comparison with the point at 30 min.

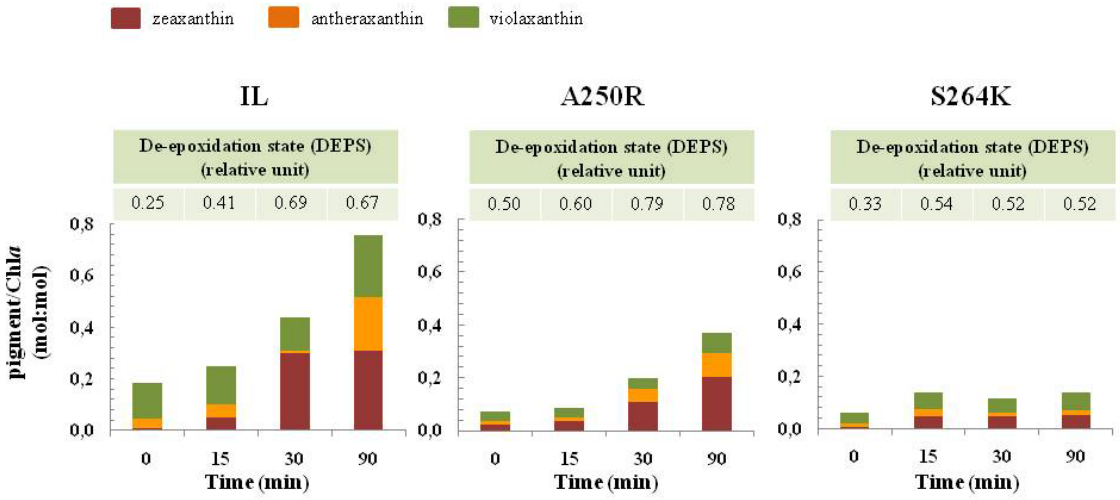



**Figure 8. Relative content of xanthophyll-cycle pigments and de-epoxidation state in D1 strains under HL/HT condition.** Violaxanthin, antheraxanthin and zeaxanthin content relative to chlorophyll a (mol:mol) in IL, A250R and S264K strains in HL/HT conditions. The standard deviation was ≤ 25 % of the means.

It seems that the A250R mutant reacted weakly to the elicitation, since small but constant increase in the relative amount of zeaxanthin (Figure 8) and lutein (Figure 9) was observed, and no response was induced in the S264K mutant. It is likely that the selected stimulus exceed the threshold for the physiological response of these strains, and particularly for S264K. The fact that the mutations in the D1 protein significantly slow down the electron transport through the PSII core complex is confirmed also by the higher DEPS in A250R at the beginning of the treatment in comparison with the reference strain.

### 4.4 Comparative analyses of time-course gene expression profiles in chlamydomonas mutants by Real-Time RT PCR in response to high light and high temperature treatments

Relative quantification of transcript levels by real-time RT-PCR has emerged over the last few years as a reliable method to study gene expression in different conditions or tissues and organisms (Guan et al., 2002).

In this study, we analysed the time-course expression profile of genes encoding enzymes involved in the biosynthesis of carotenoids (*psy*, *pds*, *lyc-β*, *lyc-ε*, *chy-β*, *zep* and *vde*),

plastoquinone (*mpbq-mt* and *hst1*) and the two PSII reaction centre proteins, D1 and D2 (*psb*A and *psb*D), in response to treatment with high light and high temperature conditions (1000 µmol photons/$m^2$/s and 37°C). Preliminary analyses included the development of an improved method to extract total RNA from *C. reinhardtii* cells as traditional technique did not satisfy our requirements in term of purity and yields. We combined different procedures, as reported in material and methods, and obtained very good results in term of RNA quality and yield (Figure 10).

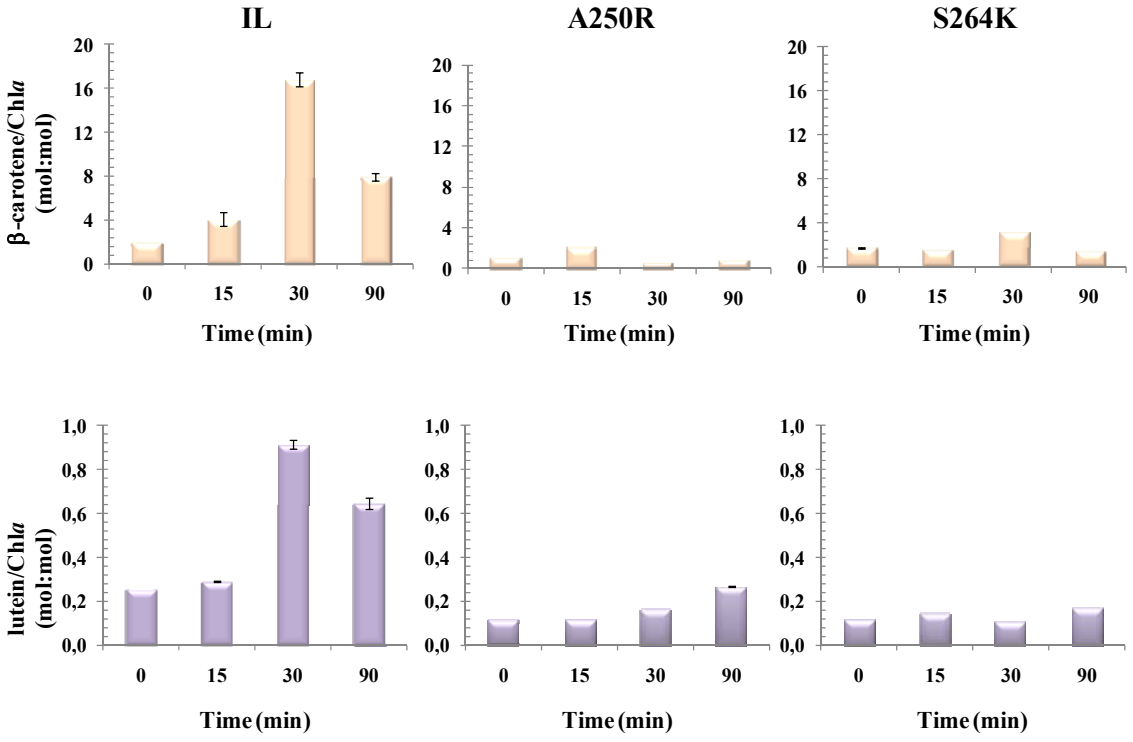



**Figure 9. Content of β-carotene relative to chlorophyll *a* in chlamydomonas strains.** Modification in β-carotene content relative to chlorophyll *a* (mol:mol) in D1 strains during control and HL/HT conditions are reported. The values represent the average (n=3) ± SD.

We, hence, designed specific primers and tested different template, primers and enzyme concentrations (see Material and methods) to optimise the real-time RT-PCR protocol. The *rack*1 gene, encoding a receptor protein of activated protein kinase C1 (RACK1), was used as the endogenous control to normalize experimental variation caused by differences in the amount of RNA added in the reverse transcription PCR reactions. The exploitation of commonly used house-keeping genes, such as *arp*4 and *arp*7 encoding for actin proteins, was excluded as their expression resulted affected during algal growth and differentially expressed in the different analysed strains (data not shown).

Results of RT-PCR analyses were expressed as fold change (FC) respect to the control untreated sample at the indicated times. A FC threshold of ± 2 was selected to describe the observed phenomena.

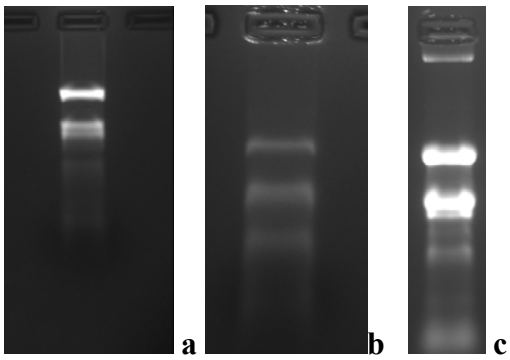



**Figure 10. Quality and quantity assessment of total RNA extracted from** *C. reinhardtii* **strains by tree different methods.** Ethidium bromide staining of rRNA extracted from culture with the same cell number: **a)** Trizol Reagent (Invitrogen), manufacturing instructions; **b)** Rneasy Plant Mini kit (Qiagen), manufacturing instructions **c)** Phenol-chloroform extraction developed in our laboratory as reported in Material and Methods.

### 4.4.1 Expression analyses of *psb*A and *psb*D genes in D1 mutants in response to high light and high temperature treatments

Light plays an important role in promoting plastid development and gene expression in photosynthetic organisms. Photoreceptors absorb light and, after the activation of the appropriate transduction pathways, promote gene regulation of nuclear-encoded genes at the transcriptional level (Deshpande et al., 1997). Regulation of chloroplast-encoded genes, however, has been shown to have a strong posttranscriptional component as in the case of the *psb*A and *psb*D genes (Mayfield et al., 1995). The chloroplastic *psb*A and *psb*D genes encode the D1 and D2 proteins, respectively, which constitute the PSII reaction centre. *psb*A regulation is the subject of intense research as the D1 protein is synthesized at very high rates in the light in order to replace photodamaged protein. Regulation of *psb*A translation is redox-regulated by photosynthetic signals, which acts at the 5' untranslated region of the mRNA, but also at the pre-RNA maturation level, increasing the splicing rate of all four introns (Deshpande et al., 1997). However, also transcriptional regulation mechanisms operate in the control of this gene in response to environmental stress (Kos et al., 2008; Luiss et al., 2006; Tyystjarvi 2002; Summerfield et al., 2008; Qian et al., 2009). Plant repair processes include the synthesis of defence proteins *via* an enhancement of gene expression to prevent the accumulation of damaged components. To determine the high light and high temperature

effects on such repair systems related to the protection of photosynthetic reactions, the gene regulation of mature *psb*A and *psb*D were analysed by real-time RT PCR (Figure 11) over a 90 min treatment. The obtained results indicated that the expression levels of *psb*D gene were un-affected by the treatment in all analysed strains. On the contrary, the *psb*A transcript levels were modulated and showed a biphasic trend of accumulation. In all analysed strains, they were transiently down-regulated after 30 min and strongly up-regulated after 90 min treatment. In particular, in IL strain the accumulation levels were higher (FC 7.4) compared to those observed in A250R (FC 5.1) and S264K (FC 3.6), reaching the highest values compared to the basal level in untreated cells. This transcription enhancement probably aims to support the high D1 protein turn over necessary to counteract the photo-oxidative damage induced by high light and high temperature treatment.

A temporarily negative and positive regulation mechanisms has been reported also for genes involved in plant defence responses (Sheng et al., 1991), strengthening the strong contribution of D1 protein turn-over in the acclimation process to high light conditions also in chlamydomonas D1 mutants.

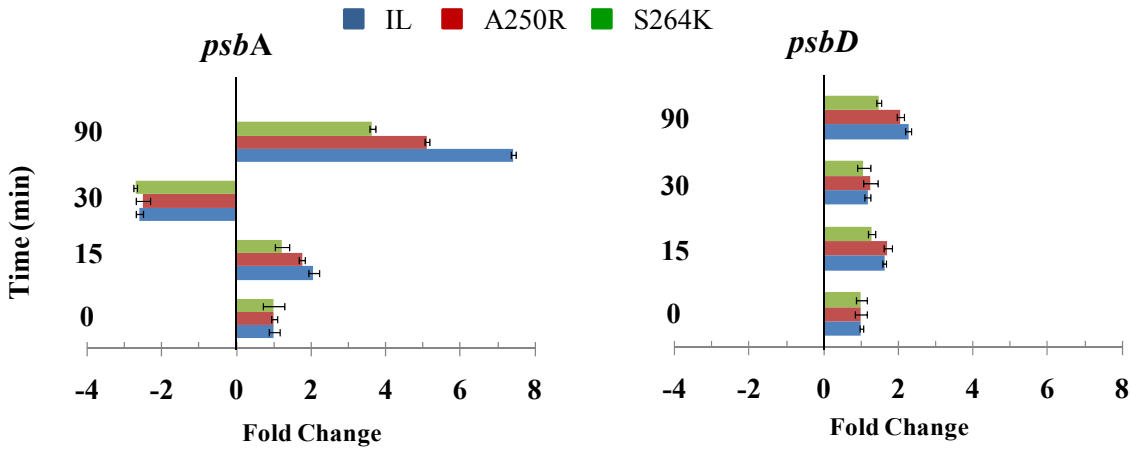



**Figure 11. Time course expression profile of *psb*A and *psb*D genes during HL/HT treatment by qRT–PCR.** Positive transcriptional regulation mechanisms were observed only for the accumulation of *psb*A mRNA. A threshold of 2-fold change was applied to express changes in relative mRNA abundance compared to control untreated samples. *rack1*gene was used as the endogenous control. The results are based on representative experiment of RNA preparation and each data point was repeated three times. Standard deviations were reported too. P≤0.05.

### 4.4.2 Expression analyses of genes involved in carotenogenisis in D1 mutant strains in response to high light and high temperature treatments

Plants have developed complex regulatory mechanisms controlling carotenoid biosynthesis and accumulation. Although there is extensive information about the main pathways involved in the synthesis of carotenoids, the control mechanisms of this route remain poorly understood. In higher plants, the transcript level of carotenogenic genes increases during fruit ripening and flower development and in response to light. In most cases transcription of carotenogenic genes are up-regulated under stress conditions (Romer et al., 2005). It is well known that heat and light stress tolerance is in part mediated by carotenoid radical scavenging capacity that protects membranes from lipid peroxidation (Davison, 2002; Havaux et al., 2007; Johnson et al., 2007). PSY, involved in the conversion of geranylgeranyl diphosphate to phytoene, and PDS, involved in the phytoene desaturation to produce ζ-carotene, have been proposed as possible limiting regulatory steps in the plastid-localized carotenoid biosynthetic pathway mediated by the nuclear-encoded corresponding genes, *psy* and *pds*. Chlamydomonas mutants deficient for PSY exhibit altered pigmentation, including a lack of coloured carotenoids and reduced levels of chlorophylls, because the absence of protective carotenoids results in photobleaching (McCarthy et al., 2004). Again, post-translational modification of *psy* mRNA levels with specific microRNAs in chlamydomonas, determined the appearance of an albino or pale green phenotype (Molnar, 2009). On the contrary, reduced expression of *pds* gene by microRNAs did not cause a reduction in the content of coloured carotenoids or a massive accumulation of phytoene (Vila et al., 2008). Phytoene desaturation was shown to be the rate limiting step of carotenogenesis in cyanobacteria, and PDS protein levels seemed to be up-regulated in arabidopsis seedlings in response to light (Wetzel and Rodermel, 1998; Chamovitz et al., 1993). In chlamydomonas, the two carotenoid genes *psy* and *pds* revealed a fast up-regulation in response to blue light, probably due to transcriptional control (Bohne and Linden, 2002; Lohr and Grossman, 2005).

In our system, exposition of *C. reinhardtii* D1 mutants to high light (1000 μmol $m^{-2}s^{-1}$) and high temperature (37°C) conditions determined significant modification at the transcriptional level of genes involved in carotenoids biosynthetic pathway. The transcript levels of *psy* was promptly induced after 15 min treatment in both IL (FC 6.3) and A250R (FC 5.4) strains, reaching the highest induction values detected over the 90 min analysed time (Figure 12). Subsequently, induced levels were strongly down-regulated at 30 min, and moderately up-regulated at 90 min. In the S264K mutant the *psy* mRNA accumulation were un-affected at 15 min and reduced 2.6 time after 30 min treatment, adopting hence, a different regulation

mechanism compared to the other strains. At the end of treatment, all the strains showed similar *psy* expression levels. The *pds* mRNA levels were transcriptionally regulated only in the control reference strains IL, being induced 3 times after 30 minutes treatment. Following, induction levels were reduced to basal levels. In the mutants A250R and S264K *pds* mRNA levels were not modified.

In addition to *psy* and *pds*, light regulation of other carotenoid genes seems plausible. Subsequent steps from the ξ-carotene production give rise to the synthesis of lycopene that is the substrate for two competing cyclases: LYCE and LYCB. On the branch starting at LYCE, successive hydroxylation and epoxidation reactions result in the synthesis of α-carotene and lutein; while on the branch starting at LYCB, β-carotenes and xanthophylls are produced (Figure 13).

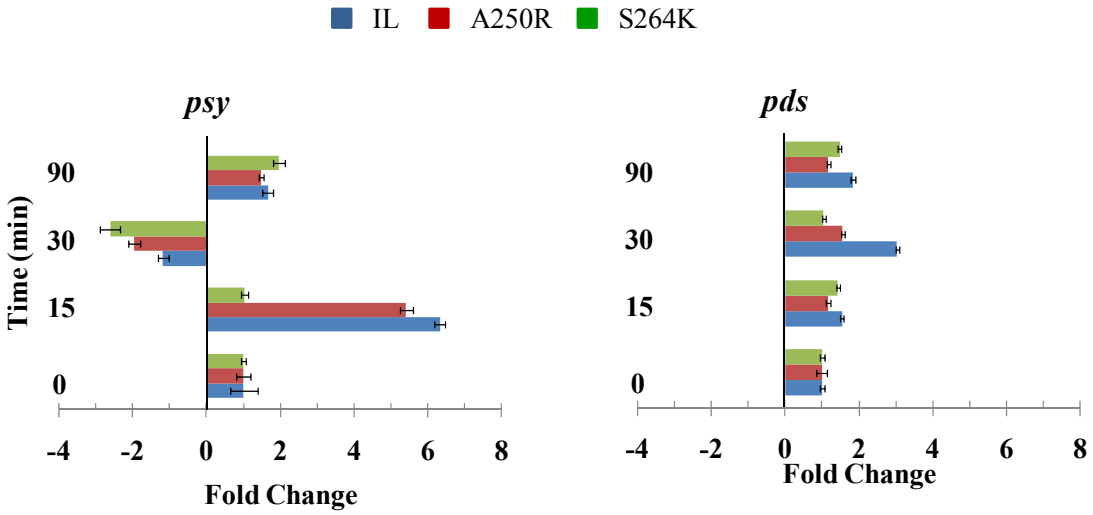



**Figure 12. Time course expression profile of *psy* and *pds* genes during HL/HT treatment by qRT–PCR.** A threshold of 2-fold change was applied to express changes in relative mRNA abundance compared to control untreated samples. *rack*1 gene was used as the endogenous control. The results are based on representative experiment of RNA preparation and each data point was repeated three times. Standard deviations were reported too. P≤0.05.

CHYB produces lutein acting on α-carotene, and zeaxanthin acting on β-carotene. Zeaxanthin in turn is epoxidated once to form antheraxanthin and twice to form violaxanthin. These reactions are reversible and catalysed by ZEP and VDE, respectively, as components of the xanthophylls cycle. Literature strongly supports the activation of both transcriptional and post-transcriptional regulation mechanisms of the corresponding genes in response to light quality and quantity, often indicating the involvement of photoreceptors in the transduction

mechanisms (Lohr and Grossmann, 2005; Grossman et al., 2004; Woitsch and Romer, 2003; Rossel et al., 2002).

In our photoinhibiting conditions, *lyc-β, lyc-ε* and *chy-β* were strongly up-regulated 15 min after treatments in all the analysed strains (Figure 13). At this time point, the highest fold changes were observed for *chy-β*, followed by *lyc-ε* and *lyc-β*. Subsequently, at 30 minutes treatment, the induced transcript levels of *chy-β* and *lyc-ε* were reduced in IL and S264K, while *lyc-β* was un-affected.

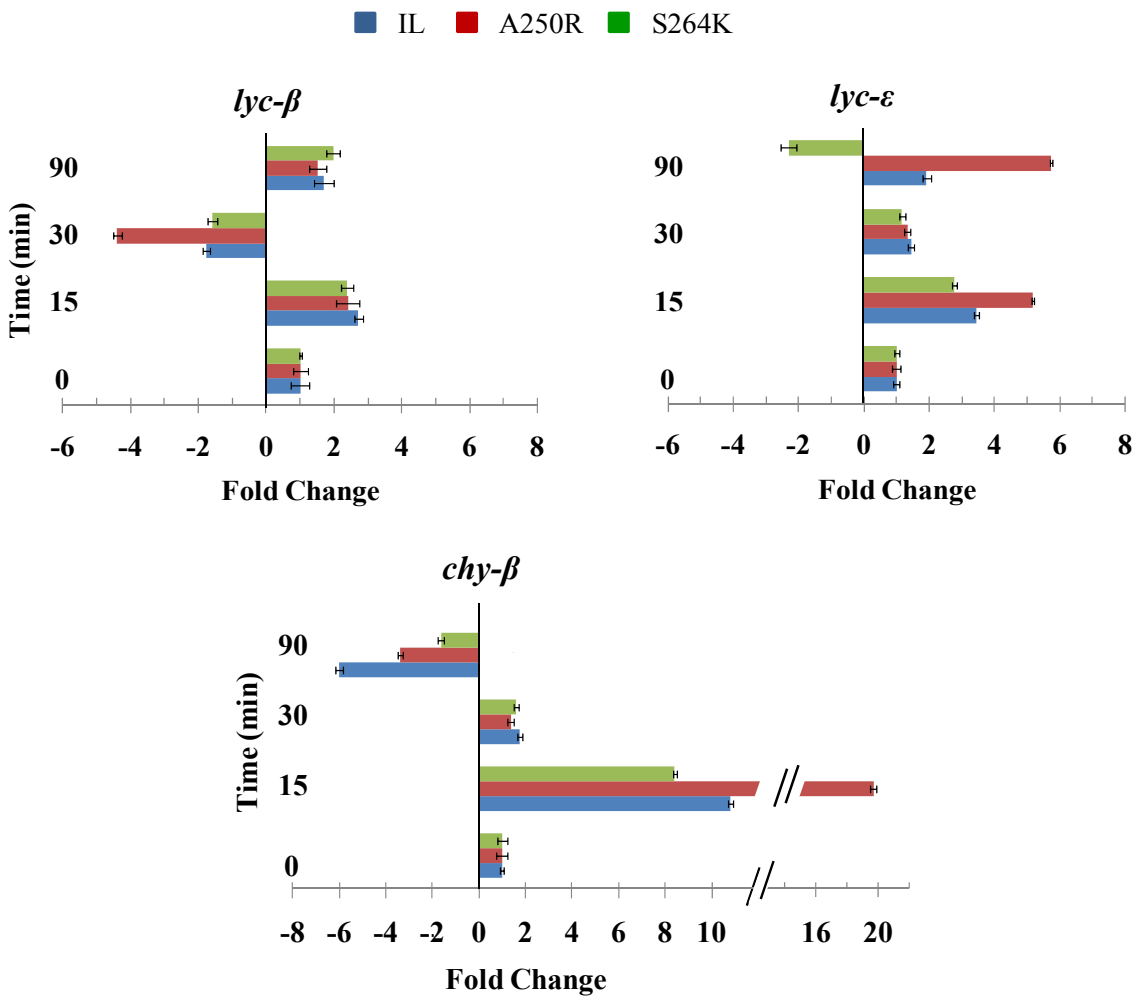



**Figure 13. Changes in relative mRNA abundance of *lyc-ε*, *lyc-β* and *chy-β* genes, involved in carotenoids biosynthetic pathway during HL/HT treatment by qRT–PCR.** A threshold of 2-fold change was applied to express changes in relative mRNA abundance compared to control untreated samples. *rack*1 gene was used as the endogenous control. The results are based on representative experiment of RNA preparation and each data point was repeated three times. Standard deviations were reported too. P≤0.05.

Conversely, in A250R strain *lyc-β* expression was strongly down-regulated (FC -4.4). Expression analyses of *chy-β* at the 90 min time point revealed no modification in S264K and a strong reduction in IL (FC -6) and A250R (FC -3.4). A strong induction of *lyc-ε* in A250R and a slight decrease in S264K were observed. *lyc-β* expression was modified only in the S264K mutant reaching two fold change induction levels. No univocal trends were observed among the different strains, indicating that specific mutations could differently affect the response to high light and high temperature exposure.

These data indicate that early regulatory mechanisms strongly induced the synthesis of *chy-β* transcript, suggesting a major need of zeaxanthin and lutein accumulation compared to β-carotene. Indeed, the zeaxanthin and lutein requirement was satisfied as evidenced by their enhanced accumulation levels observed at 30 and 90 minutes. The time lag between transcript and pigment accumulation could be explained with the *de novo* synthesis of these compounds. On the contrary, at this stage, increase in β-carotene content seems to have a lower priority for the cell, probably due to the high basal levels present in these strains. Subsequently, carotenogenic transcription profile could be modulated according to the pigments content. In fact, the higher is the accumulation of zeaxanthin and lutein, the lower is the accumulation of *chy-β* transcript at 30 and 90 minutes.

Concerning the xanthophylls interconversion pathway, we observed that expression of *vde* and *zep* genes was affected only in IL and A250R strains (Figure 14). In particular, *vde* was enhanced after 30 and 90 min treatment in IL, reaching a FC 2.4, and strongly repressed in A250R at 30 minutes with a FC of -3.1.

These data indicated that the activation of the xanthophylls cycle in IL as the VDE enzyme promotes the synthesis of zeaxanthin. Pigment profile is tightly correlated to these observations which indicated an increase of the DEPS of 2.7 fold.

On the basis of these results, we hypothesized a down-regulation of *zep* that on the contrary resulted up-regulated in IL and A250R at 90 min. This regulatory mechanism perhaps could be activated by changes in thylakoid Δ membrane pH determined as a consequence of the induced electron transport impairment.

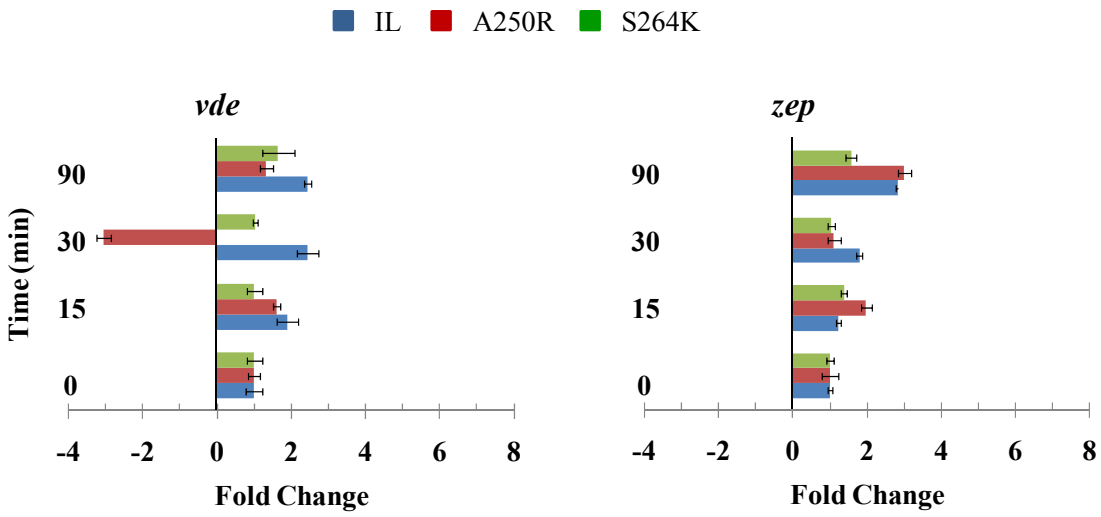



**Figure 14. Changes in relative mRNA abundance of *vde* and *zep* genes involved in carotenoids biosynthetic pathway, during HL/HT treatment by qRT–PCR.** A threshold of 2-fold change was applied to express changes in relative mRNA abundance compared to control untreated samples. *rack*1 gene was used as the endogenous control. The results are based on representative experiment of RNA preparation and each data point was repeated three times. Standard deviations were reported too. P≤0.05.

### 4.4.3 Expression analyses of genes involved in plastoquinone biosynthetic pathway in D1 mutant strains in response to high light and high temperature treatments

It has been shown that the redox state of PQ is involved in the expression of chloroplast-encoded genes (Pfannschmidt et al., 1999). PQ, as electron carrier, is in fact essential for the activity of PDS and ZDS involved in the desaturation reactions leading to carotenoid biosynthesis. These enzymes have the same reaction mechanism and use PQ as hydrogen acceptor (Norris et al., 1995; Grossman et al., 2004). If this oxidation is performed directly or indirectly by PQ is still matter of debate. Changes in PQ redox state change the trans-thylakoid pH gradient that is proposed as an indirect light intensity sensor (Wilson and Huner, 2000). Following this pathway, it influences dramatically the expression level of photosynthetic genes such as those encoding members of the *lhc* family, whose expression is strongly repressed upon exposure to strong light (Durnford et al., 2003; Maxwell et al., 1995; Escoubas et al., 1995; Wilson and Huner, 2000). Analysis of genes involved in plastoquinone biosynthesis as homogentisate solanesyl transferase (*hst1*) and methylphytyl benzoquinone methyltransferase (*mpbq-mt*), could help to understand the interaction between plastoquinone and carotenoids gene regulation. As reported above, implication of PQ redox state in carotenoids transcript regulation is well studied, while very few is known about PQ gene regulation under high light and high temperature treatments. Expression analysis showed that

*hst1* is up-regulated after 15 minutes treatment in A250R (FC 2.6) and after 30 (FC 2.5) and 90 minutes (FC 2.1) in IL strain (Figure 15). No variations in *hst1* expression have been observed in S264K strain in all analyzed time course points. Downstream to the biosynthetic pathway, the *mpbq-mt* gene was strongly repressed at 15 min in all the strains, and increased after 30 min (FC 2.4) and after 90 min, respectively, in IL (FC 3.7) and in S264K (FC 3.7). Taking into account that carotenoids and PQ biosynthesis starts from the common precursor GGPP, it is possible to hypothesise that in the early phase of the defence response the necessity of PQ is not mandatory compared to carotenoids. This is supported by the fact that carotenoids biosynthesis genes are strongly and promptly activated at 15 min, while those relative to PQ are maintained more or less at the steady state levels in the first committed step (*hst1*) (with the exception of the mutant A250R), and strongly repressed in the second one (*mpbq-mt*). Afterwards, the increasing in *mpbq-mt* mRNA levels could indicate the need of *de novo* synthesised PQ that, being oxidised, could balance the PQ over-reduction induced by the excess light intensity. In fact, PQ over-reduction leads to thylakoid lumen acidification and the literature reported this phenomenon as an important signal to induce the activation of some enzymes as VDE (Wilson and Huner, 2000; Woitsch and Romer, 2003).

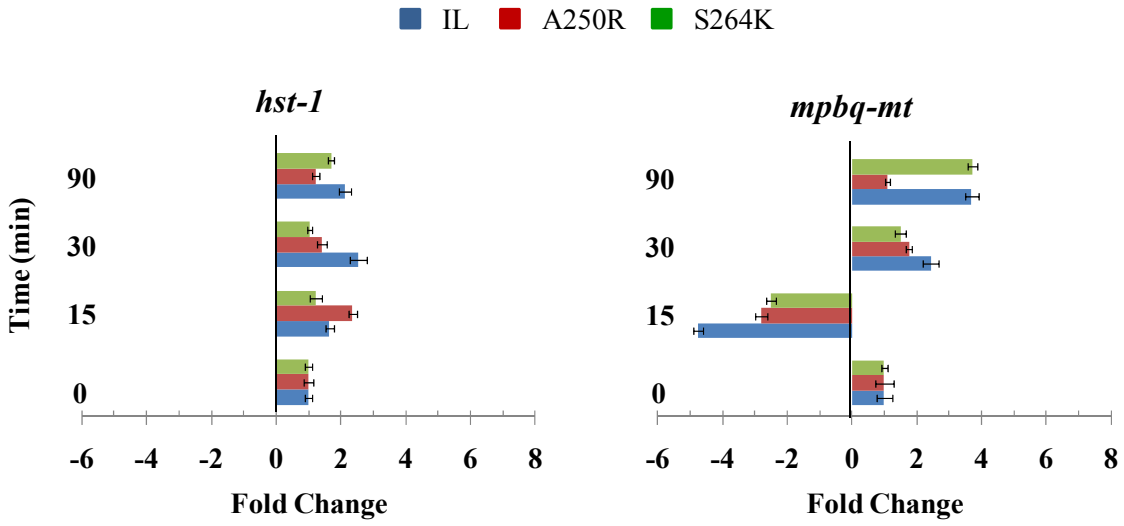



**Figure 15. Changes in relative mRNA abundance of *mpbq-mt and hst1* genes involved in plastoquinone biosynthetic pathway during HL/HT treatment by qRT–PCR.** A threshold of 2-fold change was applied to express changes in relative mRNA abundance compared to control untreated samples. rack1 gene was used as the endogenous control. The results are based on representative experiment of RNA preparation and each data point was repeated three times. Standard deviations were reported too. P≤0.05.

Often different transcript profiles were observed in A250R and S264K mutants compared to the IL reference strain. These mutants harbor aminoacidic substitution located in the binding niche of the $Q_B$ and, in the case of the S264K, the substituted amino acid is directly involved in $Q_B$ binding through hydrogen bond. It is possible that modifications at these sites could modify electron flow rate from PSII to PSI, leading to the production of different or delayed signals to the cell, in both physiological or stressful conditions.

### 4.5 *C. reinhardtii* as biological farm

In order to enhance the production of photosynthetic antioxidant pigments in *C. reinhardtii* cultures, we decided to apply extreme conditions of HL/HT (2000 μmol photons/$m^2$/s and 50°C) on IL, CC-125 and *npq2 lor1*strains. The investigation was focused on these three strains for the following reasons: the wild type IL was the biggest producer of antioxidant compounds under 1000 μmol photons/$m^2$/s and 37°C, among the D1 mutants and *npq2 lor1* mutant represents a valuable source of photosynthetic pigments. In response to the very extreme conditions superimposed to the culture, all the strains showed a significant reduction in the total content of chlorophylls, as well as a strong reduction in photosynthetic efficiency (Fv/Fm ratio) after 30 minutes of treatment (Table 5). Accumulation levels of carotenes and xanthophylls were hence quantified by HPLC.

| Time (min) | CTRL | HL/HT |
|---|---|---|
| | **Total content of chlorophylls (μg/ml)** | |
| **0** | 6.2 ± 0.55 | 6.2 ± 0.55 |
| **30** | 6.8 ± 0.45 | 5.1 ± 0.39 |
| | **Fv/Fm** | |
| **0** | 0.801 ± 0.002 | 0.801 ± 0.002 |
| **30** | 0.803 ± 0.003 | 0.212 ± 0.089 |

**Table 5. Chlorophyll content and fluorescence analyses during HL/HT treatment on *C. reinhardtii* cultures.** Reduction of the total content of chlorophylls and the maximum quantum yield of PSII photochemical reaction (Fv/Fm) in D1 strains, measured during the HL/HT experiment (2000 μmol photons/$m^2$/s and 50°C) . The values represent the average (n=3) ± SD.

Figure 16 depicts the carotenoids distribution occurring in the selected strains in physiological condition (CONTROL) and after 30 minutes treatment (TREATED) with extreme HL/HT conditions. In particular, the percentage composition of carotenes and xanthophylls is represented in parallel with the percentage composition of individual components of the

xanthophylls cycle. The elicitation effect on the carotenoids was a re-distribution of pigments in IL strain; in particular an increase of xanthophylls content (from 5.4% to 12%) and loss of β-carotene (from 94% to 88%) were observed. Violaxanthin and antheraxanthin content was not affected compared to the control.

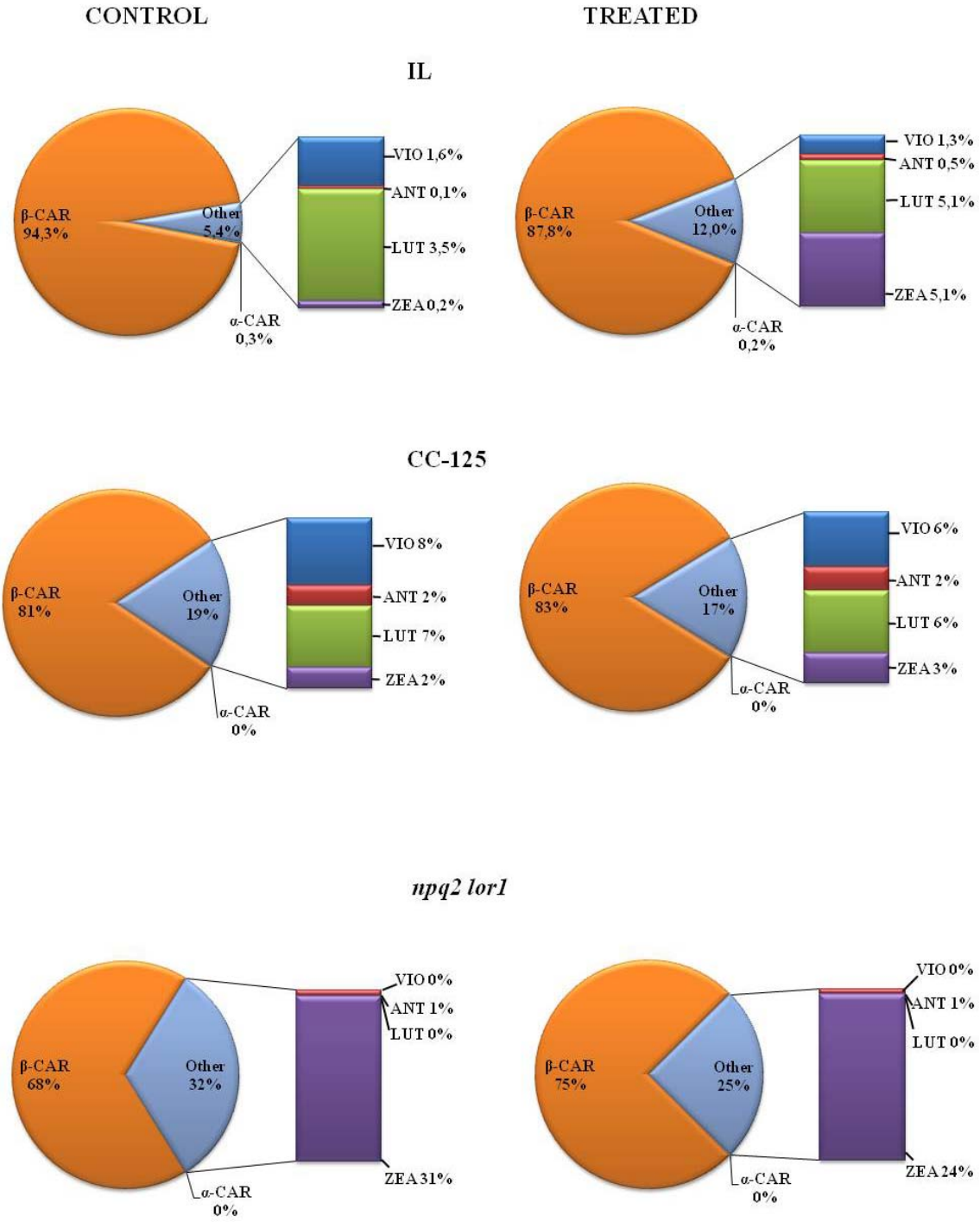



**Figure 16. Carotenoids distribution in *C. reinhardtii* strains.** Pie chart of carotenes and xanthophylls in IL, CC-125 and *npq2 lor1* strains under control and HL/HT (2000 µmol photons/$m^2$/s and 50°C) conditions.

Wild type CC-125 and the double mutant *npq2 lor1* showed a different performance in HL/HT treatment. Indeed, in wild type the carotenoids distribution was not significantly affected by the elicitation, on the contrary in *npq2 lor1* mutant an increased accumulation of β-carotene content respect to the xanthophylls pool was noted (Figure 16). Violaxanthin and antheraxanthin content was also not affected in NPQ strains.

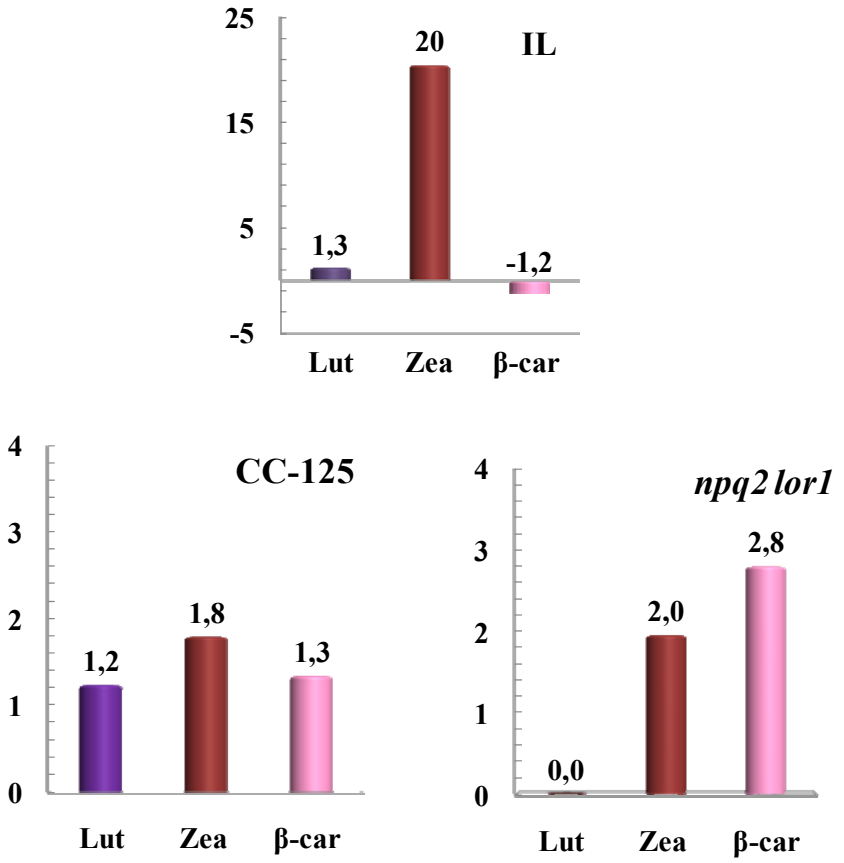



**Figure 17. Antioxidant pigment accumulation after elicitation in *C. reinhardtii* strains.** Accumulation levels of β-carotene, zeaxanthin and lutein after elicitation (2000 μmol photons/$m^2$/s and 50°C) in IL, CC-125 and *npq2 lor1* strains. The values represent the pigment fold change compared to the control content.

Figure 17 depicted the fold change of β-carotene, zeaxanthin and lutein accumulation compared to the control content (ng of pigment/mg of dry weight). In all analysed strains a general accumulation of antioxidant compounds was noted, even if the maximum fold change was observed for zeaxanthin accumulation in IL (FC 20). Moreover, in IL strain a decrease in the β-carotene profile was observed (FC -1.2). We hypothesized that the excess of energy in stressful conditions led to a strong request of zeaxanthin and hence to the β-carotene consumption according to the literature (Haghjou et al., 2008). As a whole these results indicated that in these conditions, the IL strain seems to be the most responsive to the applied

stress, strengthening the potentiality of this strain to be modulated in order to modify its metabolisms for zeaxanthin production. On the contrary, *npq2 lor1* mutant has a major zeaxanthin and β-carotene content, but is less susceptible to metabolism modification. As a consequence, among selected strains, for nutraceutical purposes, we identify *npq2 lor1* mutant as the best producer of antioxidants pigments.

# 5. CONCLUSIONS

In this PhD thesis, *C. reinhardtii* wild type strains and mutants in the D1 protein and in the enzymes involved in carotenoids biosynthetic pathway were investigated. Our aim was to study the PSII performance in physiological and stressful conditions. It was shown that aminoacid substitution in the D1 protein located in the $Q_B$ binding site remarkably reduced the efficiency of the electron transfer between the primary ($Q_A$) and the secondary ($Q_B$) quinone acceptors in PSII following the order IL>A250R>S264K. This finding suggested that the D1 aminoacid substitution of Arg in position 250 and Ser in position 264 straightforward altered the photosynthetic electron transfer rate reducing the PSII efficiency. This reduction is reflected in the lower oxygen evolution capacity of the D1 mutants compared to the reference strain IL. Moreover, the different Chl*a*/cell ratios between D1 strains, which are indicative of a reduced peripheral antenna cross section, suggested possible modifications in D1 sovra-molecular structure and modifications in the PSII architecture of A250R and S264K mutants.

Also the photosynthetic pigment quantification was different among strains, mainly in the content of the total xanthophylls e.g. violaxanthin, anteraxanthin, zeaxanthin and lutein content. Comparison of the pigment profiles of all the analysed strains showed a notably higher pigments content in NPQ mutants compared to the D1 strains (*npq2 lor1*> *npq2*>*lor1*>CC-125>IL, A250R, S264K). These results suggested that under physiological conditions the best producers of antioxidant photosynthetic pigments among the D1 strains was the wild type IL, and among the NPQ strains the *npq2 lor1* mutant.

In order to enhance the content of antioxidant photosynthetic pigments in *C. reinhardtii* cultures for nutraceutical purposes, we applied a set of abiotic stresses capable to induce oxidative stress. Among these, superimposition of 1000 μmol of photons/$m^2$/s and 37°C led in all analysed D1 strains a strong reduction in photosynthetic efficiency (measured as Fv/Fm ratio), without affecting the total chlorophyll content. Pigment profile highlighted the noteworthy response of the reference strain IL in comparison with D1 mutants. In fact, after 30 and 90 minutes of treatment, a great lutein and zeaxanthin accumulation in IL strain was observed, as well as an activation of xanthophylls cycle.

In the pioneering background that describes the unicellular microalgae as natural source for valuable compounds, a second set of elicitation experiment was performed on IL, CC-125 and *npq2 lor1* strains to achieve a biggest accumulation of antioxidant compounds under extreme high light and high temperature conditions (2000 μmol of photons/$m^2$/s and 50°C).

The reported data showed that IL strain seems to be the most responsive to the stress, for its aptitude to modulate its metabolism for zeaxanthin production. On the contrary, *npq2 lor1*

mutant has a major zeaxanthin and β-carotene content, but it is less susceptible to metabolism modification. As a consequence, among selected strains, for nutraceutical purpose, we identified *npq2 lor1* mutant as the best producer of antioxidants pigments.

In order to realize future metabolic engineering projects and gain further insights in the elucidation of the defence mechanisms underlying the high light/high temperature response, we performed by real-time RT-PCR the relative quantification of transcript levels of genes involved in the carotenoids biosynthesis (*psy, pds, lyc-β, lyc-ε, chy-β, zep* and *vde*), plastoquinone (*mpbq-mt* and *hst1*) and the two PSII reaction centre proteins, D1 and D2 (*psb*A and *psb*D).

The early activation of *chy-β, lyc-β, lyc-ε* and *psy* genes suggested that the main algae necessity in stressful condition was the induction of carotenoids production. Subsequently, the requirement of oxidised PQ synthesis, become important. This was indicated by the over expression of *mpbq-mt* gene. In addition, *mpbq-mt* and *pds* gene induction levels were temporarily correlated, indicating the involvement of PQ in carotenoids biosynthesis (Figure 18). It has been proposed that the redox state of PQ and the consequent acidification of the thylakoids lumen, are involved in the expression of chloroplast-encoded genes (Pfannschmidt et al., 1999). Our data strongly support these observations. It is known that PQ acts in electron transport chain during photosynthesis light-dependent reactions. The ratio between reduced and oxidized plastoquinone is hence a good indicator of the balance between light absorption and electron transport rate. When the adsorbed light exceeds the PSII energy capacity, PQ become over reduced. As a consequence, the PQ pool redox state behaves as a key signal modulating the expression of photosynthesis-related genes in response to light conditions and in some cases it maybe acting directly on the dedicated enzymes.

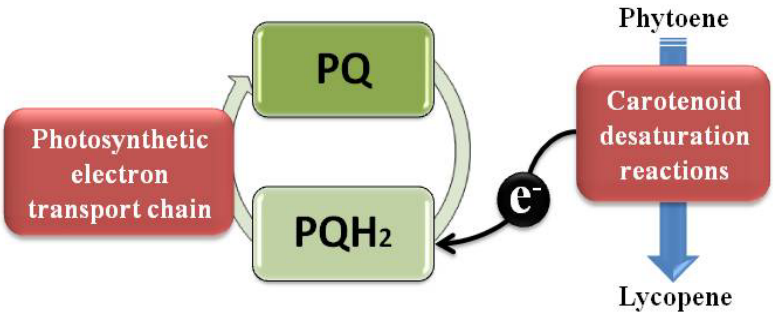



**Figure 18. Model for the involvement of PQ in carotenoid biosynthetic pathway.** Proposed interactions of plastoquinone, carotenoid desaturation enzymes, and the photosynthetic electron transport chain. Adapted by Norris et al., 1995.

Future work will be dedicated to further clarify the involvement of PQ redox state in carotenogenesis pathway by either proteomic or microRNA approaches. In addition, taking into consideration the relevance of the lipid environment in the light and heat stress response, we will determine the lipid profile of different chlamydomonas mutants in these stressful conditions, in order to highlight and correlate structural and compositional modifications to the tolerance mechanisms.

For nutraceutical purposes, chlamydomonas growth conditions will be set up for large scale production of biomass with high nutraceutics content, and a safe protocol extraction, possibly by the exploitation of supercritical fluids technology, will be developed. In addition, as *C. reinhardtii* belongs to the category of organism having a GRAS status, it could be used as food ingredient without involving costly and complex isolation and purification procedures. This could provide a cost-effective means, obviously only after application of EU directives on novel *food* and EU approval.

## 6. REFERENCES

**Adam Z, Rudella A, van Wijk KJ.** (2006). Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts. *Curr Opin Plant Biol;* **9:**234–240.

**Adir N, Zer H, Shochat S, Ohad I.** (2003). Photoinhibition an historical perspective. *Photosynth Res;* **76:**343–370.

**Albrecht M, Misawa N, Sandmann G.** (1999). Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids beta-carotene and zeaxanthin. *Biotechnology letters*; **21**: 791-795.

**Allen JF.** (2004). Cytochrome $b_6f$: Structure for signalling and vectorial metabolism. *Trends Plant Science*; **9:** 130–137.

**Andersen JK.** (2004(. Oxidative stress in neurodegeneration: cause or consequence? *Nature Reviews Neuroscience*; **5:** S18–S25.

**Anwaruzzaman M, Chin BL, Li X-P, Lohr M, MartinezDA, Niyogi KK.** (2004). Genomic analysis of mutants affecting xanthophyll biosynthesis and regulation of photosynthetic light harvesting in *Chlamydomonas reinhardtii*. *Photosynthesis Research;* **82(3)**: 265:276.

**Apel K and Hirt H.** (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol;* **55:**373–399.

**Ariga T and Seki T.** (2006). Antithrombotic and anticancer effects of garlic-derived sulfur compounds: a review. *Biofactors*; **26(2):** 93-103.

**Aro EM, Suorsa M, Rokka A, Allahverdiyeva Y, Paakkarinen V, Saleem A et al** (2005). Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. *J Exp Bot;* **56:**347–356.

**Aro EM, McCaffery S, Anderson JM.** (1993). Photoinhibition and D1 protein degradation in peas acclimated to different growth irradiances. *Plant Physiology;* **103**: 835-843.

**Asada K.** (1996). Radical production and scavenging in the chloroplasts. In: Baker NR (ed) Photosynthesis and the environment. *Kluwer Academic Publishers, Dordrecht, The Netherlands*, pp.123–150.

**Barber J.** (2006) Photosystem II: an enzyme of global significance. *Biochemical Society transactions;* **34(5):**619-31.

**Barber J.** (1982). Influence of surface charges on thylakoid structure and function. *Annu Rev Plant Physiol;* **33:**261–295.

**Barber J and Andersson B.** (1992). Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem Sci;* **17:**61–66.

**Barnes D, Franklin S, Schultz J, Henry R, Brown E, Coragliotti A, Mayfield SP.** (2005). Contribution of 5'- and 3'-untranslated regions of plastid mRNAs to the expression of *Chlamydomonas reinhardtii* chloroplast genes. *Molecular Genetics and Genomics*; **274**: 625-636.

**Baroli I, Do AD, Yamane T, Niyogi KK.** (2003). Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects *Chlamydomonas reinhardtii* from photooxidative stress. *Plant Cell;* **15**: 992–1008.

**Barrie Margetts and Judith Buttriss** (2003). Plants: Diet and Health. *Gail Goldberg, Dr. (Editor), BNF (British Nutrition Foundation) Wiley-Blackwell*.

**Barzegari A, Hejazi MA, Hosseinzadeh N, Eslami S, Mehdizadeh Aghdam E, Hejazi MS.** (2009). Dunaliella as an attractive candidate for molecular farming. *Molecular Biology Reports*; *in press*.

**Bassi R, Sandona D, Croce R**. (1997). Novel aspects of chlorophyll *a/b*-binding proteins. *Physiol Plant;* **100:** 769–779.

**Bateman JM and Purton S.** (2000). Tools for chloroplast transformation in Chlamydomonas: expression vectors and a new dominant selectable marker. *Molecular & general genetics*; **263**: 404-10.

**Baur JA and Sinclair DA.** (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*; **5(6):** 493-506.

**Ben-Amotz A.** (1996). Effect of low temperature on the stereoisomer composition of *β*-carotene in the halotolerant alga *Dunaliella bardawil* (Chlorophyta). *Journal of Phycology;* **32**: 272–75.

**Benz CC and Yau CA.** (2008). Oxidative stress and cancer: paradigms in parallax. *Nat Rev Cancer*; **8:** 875–879.

**Beyer P, Al-Babili S, Ye X, Lucca P, Schaub P, Welsch R and Potrykus I.** (2002). Golden rice: Introducing the β-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. *The Journal of nutrition*; **132(3):** 506S-510S.

**Björkman O and Demmig B.** (1987). Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins. *Planta;* **170**: 489-504.

**Block G, Patterson B, Subar A.** (1992). Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutrition and cancer* **18**: 1-29.

**Blowers AD, Ellmore GS, Klein U, Bogorad L.** (1990). Transcriptional analysis of endogenous and foreign genes in chloroplast transformants of Chlamydomonas. *Plant Cell*; **2**:1059-1070.

**Blowers AD, Bogorad L, Shark KB, Sanford JC.** (1989). Studies on Chlamydomonas chloroplast transformation: foreign DNA can be stably maintained in the chromosome. *Plant Cell*; **1**: 123-132.

**Bohne F and Linden H.** (2002). Regulation of carotenoid biosynthesis genes in response to light in *Chlamydomonas reinhardtii*. *Biochimica and Biophysica Acta;* **1579**: 26–34.

**Bonk M, Hoffmann B, Von Lintig J, Schledz M, Al-Babili S, et al.** (1997). Chloroplast import of four carotenoids biosynthetic enzymes in vitro reveals differential fates prior to membrane binding and oligomeric assembly. *European Journal of Biochemistry;* **247**: 942–50.

**Boussiba S.** (2000). Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiol. Plant;* **108:**111–17.

**Bouvier F, Keller Y, d'Harlingue A, Camara B.** (1998). Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L.). *Biochimica et Biophysica Acta;* **1391**: 320–28.

**Bouvier F, d'Harlingue A, Hugueney P, Marin E, Marion-Poll A, Camara B.** (1996). Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *The Journal of Biological Chemistry;* **271**: 28861–67.

**Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM and Shark KB et al.** (1988). Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science*; **240**: 1534-1538.

**Breitenbach J, Misawa N, Kajiwara S, Sandmann G.** (1996). Expression in *Escherichia coli* and pro-perties of the carotene ketolase from *Haematococcus pluvialis*. *FEMS Microbiology Letters;* **140**: 241–46.

**Britton G.** (1998). Overview of carotenoid biosynthesis. Britton G, Liaaen-Jensen S, Pfander H, eds. 1998. *Carotenoids: Biosynthesis and Metabolism*. Basel: Birkhäuser, pp. 13–147

**Brown L, Rimm EB, Seddon JM, Giovannucci EL, Chasan-Taber L, Spiegelman D, Willett WC and Hankinson SE.** (1999). A prospective study of carotenoid intake and risk of cataract extraction in US men. *The American journal of clinical nutrition*; **70**:517-524.

**Bugos RC, Hieber AD, Yamamoto HY.** (1998). Xanthophyll cycle enzymes are members of the lipocalin family, the first identified from plants. *The Journal of Biological Chemistry;* **273**: 15321–24.

**Bugos RC, Yamamoto HY.** (1996). Molecular cloning of violaxanthin de-epoxidase from romaine lettuce and expression in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America;* **93**: 6320–25.

**Bukhov NG and Mohanty P. (1999).** Elevated temperature stress effects on photosystems: characterization and evaluation of the nature of heat induced impairments. In: G. S. Singhai, G. Renger, S. K. Sopory, K. D. Irrgang, Govindjee (Eds), Concepts in Photobiology: Photosynthesis and Photomorphogenesis, Narosa Publishing House, New Delhi, India, pp. 617-648.

**Bumann D and Oesterhelt D.** (1995). Destruction of a single chlorophyll is correlated with the photoinhibition of photosystem II with a transiently inactive donor side. *Proceedings of the National Academy of Sciences of the United States of America*; **92**: 12195-12199.

**Burkhardt PK, Beyer P, Wünn J, Klöti A, Armstrong GA, Schledz M, von Lintig J, Potrykus I.** (1997). Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *The Plant journal*; **11**: 1071-1078.

**Caffarri S, Croce R, Breton J, Bassi R.** (2001). The major antenna complex of Photosystem II has a xanthophyll binding site not involved in light harvesting. *J Biol Chem* **276**: 35924–35933.

**Cai Y, Wang R, Pei F, Liang BB.** (2007).Antibacterial activity of allicin alone and in combination with bold beta-lactams against *Staphylococcus spp*. and *Pseudomonas aeruginosa*. *J Antibiot*; **60:** 335-338.

**Callahan FE, Becker DW, Cheniae GM.** (1986). Studies on the photoinactivation of the water-oxidizing enzyme. II. Characterization of weak light photoinhibition of PS II and its light-induced recovery. *Plant Physiol;* **82:**261–269.

**Carol P abd Kuntz M.** (2001). A plastid terminal oxidase comes to light: implications for carotenoid biosynthesis and chlororespiration. *Trends in Plant Science;* **6**: 31–36.

**Cermak R.** (2008). Effect of dietary flavonoids on pathways involved in drug metabolism. *Expert Opin Drug Metab Toxicol*; **4(1):** 17-35.

**Chamovitz D, Sandmann G, Hirschberg J.** (1993). Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis. *The Journal of biological chemistry;* **268**: 17348– 17353.

**Chasan-Taber L, Willett WC, Seddon JM, Stampfer MJ, Rosner B, Colditz GA, Speizer FE and Hankinson SE.** (1999). A prospective study of carotenoid and vitamin A intakes and risk of cataract extraction in US women. *The American journal of clinical nutrition*; **70**: 509-516.

**Chew BP, Brown CM, Park JS, Mixter PF.** (2003). Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis. *Anticancer research*; **23**: 3333-3339.

**Chitchumroonchokchai C, Bomser JA, Glamm JE, Failla ML.** (2004). Xanthophylls and α-tocopherol decrease UVB-induced lipid peroxidation and stress signalling in human lens epithelial cells. *The Journal of nutrition*; **134:** 3225-3232.

**Corona V, Aracri B, Kosturkova G, Bartley GE, Pitto L, Giorgetti L, Scolnik PA, Giuliano G.** (1996). Regulation of a carotenoid biosynthesis gene promoter during plant development. *Plant journal* **9**: 505–512.

**Cowan AK, Logie MRR, Rose PD, Phillips LG.** (1995). Stress induction of zeaxanthin formation in the caroteneaccumulating alga *Dunaliella salina* Teod. *Journal of Plant Physiology;* **146**:554–62.

**Crofts AR.** (2004). The Q-cycle-a personal perspective. *Photosynthesis Research*; **80(1-3):**223-43.

**Cunningham FX Jr and Gantt E.** (2001). One ring or two? Determination of ring number in carotenoids by lycopene epsiloncyclases. *Proceedings of the National Academy of Sciences of the United States of America;* **98**: 2905–10.

**Cunningham FX Jr and Gantt E.** (1998). Genes and enzymes of carotenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology;* **49**: 557–83.

**Cunningham FX Jr, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E.** (1996). Functional analysis of the $\beta$ and $\varepsilon$ lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell;* **8**: 1613–26.

**Cushman JC and Bohnert HJ.** (2000). Genomic approaches to plant stress tolerance. *Current Opinion in Plant Biology;* **3**: 117–124.

**Cysewski GR and Lorenz RT.** (2004). Industrial production of microalgal cell-mass and secondary products—species of high potential. Haematococcus. In: Richmond A (ed) Handbook of microalgal culture. *Biotechnology and applied phycology. Blackwell Science, Oxford, UK;* **pp**: 281–288.

**Daniell H, Khan MS, Allison L.** (2002). Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *TRENDS in Plant Science*; **7**: 84-91.

**Daniell H, Datta R, Varma S, Gray S and Lee S.** (1998). Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nature biotechnology*; **16**: 345-3.

**Davison PA.** (2002). Overexpression of beta-carotene hydroxylase enhances stress tolerance in Arabidopsis. *Nature;* **418**: 203–206.

**Debuchy R, Purton S, Rochaix JD.** (1989). The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. *The EMBO journal*; **8**: 2803-2809.

**Del Campo JA, García-González M, Guerrero MG.** (2007). Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Applied Microbiology and Biotechnology*; **74(6)**: 1163-74.

**DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H.** (2001). Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiology*; **127(3)**: 852-62.

**Derguini F, Mazur P, Nakanishi K, Starace DM, Saranak J, Foster KW.** (1991). All-trans-retinal is the chromophore bound to the photoreceptor of the alga *Chlamydomonas reinhardtii*. *Photochemistry and Photobiology*; **54**: 1017–21.

**Demmig-Adams B and Adams WW III.** (2009). Overview of diet-gene interactions and the example of xanthophylls. In: MT Giardi, G Rea and B Berra editors. Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors. LANDES BioScience and Springer, Publisher, in press.

**Demmig-Adams B and Adams WW III.** (2006). Photoprotection in an ecological context: the remarkable complexity of thermal dissipation. *The New phytologist*; **172**: 11-21.

**Demmig-Adams B and Adams WW III.** (2002). Antioxidants in photosynthesis and human nutrition. *Science*; **298**: 2149-2153.

**Demmig-Adams B.** (2003). Linking the xanthophyll cycle with thermal energy dissipation. *Photosynthesis Research;* **76:** 73–80.

**Demmig-Adams B and Adams WW III.** (1996). The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in plant science*; **1**: 21-26.

**Demmig-Adams B, Adams WW, Heber U, Neimanis S, Winter K, Krüger A, Czygan FC, Bilger W, Björkman O.** (1990). Inhibition of zeaxanthin formation and of rapid changes in radiationless energy dissipation by dithiothreitol in spinach leaves and chloroplasts. *Plant Physiol*; **92(2):**293-301.

**Derguini F, Mazur P, Nakanishi K, Starace DM, Saranak J, Foster KW.** (1991). All-trans-retinal is the chromophore bound to the photoreceptor of the alga *Chlamydomonas reinhardtii*. *Photochem. Photobiol;* **54:**1017–21.

**Deshpande NN, Bao Y, Herrin DL.** (1997). Evidence for light:redoxregulated splicing of *psbA* pre-RNAs in *Chlamydomonas* chloroplasts. *RNA;* **3**: 37–48.

**Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G.** (2002). Metabolic engineering of xanthophylls content in tomato fruits. *FEBS Letters*; **519**: 30-34.

**Diner BA and Rappaport F.** (2002). Structure, dynamics, and energetics of the primary photochemistry of photosystem of oxygenic photosynthesis. *Annual Review of Plant Biology,* **53:** 551-580.

**Diner BA, Reis DF, Cohen BN, Metz JG.** (1988) COOH-terminal processing of polypeptide D1 of the photosystem II reaction center of *Scenedesmus obliquus* is necessary for the assembly of the oxygen-evolving complex. *J Biol Chem;* **263:**8972–8980.

**Ducreux LJ, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MA.** (2005). Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *Journal of experimental botany*; **56**: 81-89.

**Durnford DG, Price JA, Mckim SM, and Sarchfield ML.** (2003). Light-harvesting complex gene expression is controlled by both transcriptional and post-transcriptional mechanisms during photoacclimation in *Chlamydomonas reinhardtii*. *Physiologia Plantarum* **118**: 193–205.

**Eberhard S, Drapier D, Wollman FA.** (2002). Searching limiting steps in the expression of chloroplast encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*. *The Plant Journal*; **31**: 149-160.

**Edelman M and Mattoo AK.** (2008). D1-protein dynamics in photosystem II: the lingering enigma *Photosynth Res;***98(1-3):**609-20.

**Ellinger S, Ellinger J, Stehle P.** (2006). Tomatoes, tomato products and lycopene in the prevention and treatment of prostate cancer: do we have the evidence from intervention studies? *Current opinion in clinical nutrition and metabolic care*; **9**:722-727.

**Ellstrand NC.** (2003). Current knowledge of gene flow in plants: implications for transgene flow. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*; **358**: 1163-70.

**Ellstrand NC.** (2001). When transgenes wander, should we worry? *Plant Physiol*; **125**: 1543-5.

**Emanuelsson A, Eskling M, Akerlund HE.** (2003). Chemical and mutational modification of histidines in violaxanthin de-epoxidase from *Spinacia oleracea*. *Physiologia Plantarum;* **119**: 97–104.

**Ferris PJ.** (1995). Localization of the nic-7, ac-29 and thi-10 genes within the mating-type locus of *Chlamydomonas reinhardtii*. *Genetics*; **141**: 543-549.

**Fiamegos CG N, Vervoort J, Stalikas CD.** (2004). Analytical procedure for the in-vial derivatization extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass-selective detection. *J Chromatogr A*; **1041:** 11-18.

**Fischer BB, Wiesendanger M, Eggen RIL.** (2006). Growth condition-dependent sensitivity, photodamage and stress response of *Chlamydomonas reinhardtii* exposed to high light conditions. *Plant Cell Physiology;* **47(8)**: 1135–1145.

**Formaggio E, Cinque G, Bassi R.** (2001). Functional architecture of the major light-harvesting complex from higher plants. *Journal of Molecular. Biology;* **314:** 1157–1166.

**Foyer CH and Noctor G.** (2003). Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol Plant;* **119:**355–3.

**Franklin S, Ngo B, Efuet E, Mayfield SP.** (2002). Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal*; **30**: 733-744.

**Fraser PD and Bramley PM.** (2004). The biosynthesis and nutritional uses of carotenoids. Progress in *Lipid Research*; **43**: 228–265.

**Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, et al.** (2002). Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit specific manner. *Proceedings of the National Academy of Sciences of the United States of America;* **99**: 1092–97.

**Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, et al.** (1995). Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *The Plant Journal;* **8**: 693–701.

**Fujisawa M, Watanabe M, Choi SK, Teramoto M, Ohyama K, Misawa N.** (2008). Enrichment of carotenoids in flaxseeds by metabolic engineering with introduction of bacterial phytoene synthase gene crtB. *Journal of bioscience and bioengineering*; **105**: 636-641.

**Garcìa-Lorenzo M, Zelisko A, Jackowski G, Funk C.** (2005). Degradation of the main photosystem II light-harvesting complex. *Photochem Photobiol Sci;* **4:**1065–1071.

**Gastaldelli M, Canino G, Croce R, Bassi R.** (2003). Xanthophyll binding sited of the CP29 (Lhcb4) subunit of higher plant Photosystem II by domain swapping and mutant analysis. *J Biol Chem* **278**: 19190–19198.

**Ghirardi ML, Mahajan S, Sopory SK, Edelman M, Mattoo AK.** (1993). Photosystem II reaction center particle from Spirodela stroma lamellae. *J Biol Chem;* **268:**5357–5360.

**Ghirardi ML, Callahan FE, Sopory SK, Elich TD, Edelman M, Mattoo AK.** (1990). Cycling of the photosystem II reaction center core between grana and stromal lamellae. In: Baltscheffsky M (ed) *Current research in photosynthesis*, vol 2. Kluwer, Dordrecht, pp 733–738.

**Gilmore AM.** (1997). Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. *Physiol Plant;* **99:** 197–209.

**Gilmore AM and Yamamoto HY.** (1991). Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. *J Chromatogr;* **543:** 137–145.

**Giuliano G, Tavazza R, Diretto G, Beyer P, Taylor MA.** (2008). Metabolic engineering of carotenoid biosynthesis in plants. *Trends in Biotechnology*; **26**: 139–145.

**Giuliano G, Bartley GE, Scolnik PA** (1993). Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* **5**: 379–387.

**Glade MJ. (**1999). Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. Nutrition Jun; **15(6)**: 523-6.

**Goldschmidt-Clermont M.** (1991). Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of Chlamydomonas. *Nucleic acids research*; **19(15)**: 4083-9.

**Goodenough UW.** (1992) Green yeast. *Cell*; **70(4):**533-8.

**Green BR and Durnford D.** (1996). Chlorophyll-carotenoid proteins in oxygenic photosynthesis. *Annual. Review Plant Physiology*, *Plant Molecular Biology;* **47:**685-714.

**Griesbeck C, Kobl I, Heitzer M.** (2006). *Chlamydomonas reinhardtii*: a protein expression system for pharmaceutical and biotechnological proteins. Molecular Biotechnology; **34**: 213-23.

**Grossman AR, Lohr M, Im CS.** (2004). *C. reinhardtii* in the landscape of pigments. *Annual*

*Review of Genetics*; **38**:119–73.

**Grossman AR, Harris EE, Hauser C, Lefebvre PA, Martinez D, Rokhsar D, Shrager J.** (2003). Chlamydomonas reinhardtii at the crossroads of genomics. *Eukaryot Cell.;* **2(6):**1137-50.

**Hager A, Holocher K.** (1994). Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. *Planta;* **192**: 581–89.

**Harris EH.** (2001). Chlamydomonas as a Model Organism. *Annual review of plant physiology and plant molecular biology*.; **52**: 363-406.

**Hartmann, R, Meisel, H.** (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology*; **18**: 163-169.

**Havaux M, Dall'Osto L, Bassi R.** (2007). Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII antennae. *Plant Physiology*; **145**: 1506–1520.

**Havaux M, Dall'Osto L, Cuiné S, Giuliano G and Bassi R.** (2004). The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana. The Journal of biological chemistry*; **279**: 13878-13888.

**Havaux M and Niyogi KK.** (1999). The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proceedings of the National Academy of Sciences of the United States of America*

*USA*; **96**: 8762-8767.

**Hermsmeier D, Mala E, Schulz R, Thielmann J, Galland P, Senger H.** (1991). Antagonistic blue- and red-light regulation of cab-gene expression during photosynthetic adaptation in *Scenedesmus obliquus*. *Journal of photochemistry and photobiology Biology* **11**: 189–202.

**Higuera-Ciapara I, Félix-Valenzuela L, Goycoolea FM.** (2006). Astaxanthin: a review of its chemistry and applications. *Critical Reviews in Food Science and Nutrition*; **46**: 185–196.

**Hirayama S, Ueda R, Sugata K.** (1995). Effect of hydroxyl radical on intact microalgal photosynthesis. *Energy Convers Manag;* **36:**685–688.

**Hirschberg J.** (2001). Carotenoid biosynthesis in flowering plants. *Current Opinion in Plant Biology;* **4**: 210–18.

**Hirschberg J.** (1998). Molecular biology of carotenoid biosynthesis. Britton G, Liaaen-Jensen S, Pfander H, eds. 1998. *Carotenoids: Biosynthesis and Metabolism*. Basel: Birkhäuser, pp. 149–94.

**Holt NE, Zigmantas D, Valkunas L, Li XP, Niyogi KK, Fleming GR.** (2005). "Carotenoid cation formation and the regulation of photosynthetic light harvesting". *Science*. 21;**307(5708):**433-6.

**Holt NE, Fleming GR, Niyogi KK.** (2004) Toward an understanding of the mechanism of the nonphotochemical quenching in green plants. *Biochemistry*. **43:** 8281–8289.

**Hope AB.** (2000). Electron transfers amongst cytochrome f, plastocyanin and photosystem I: kinetics and mechanisms. *Biochimica et Biophysica Acta*. **1456(1):**5-26.

**Horton P, Ruban, AV, Walters RG.** (1996). Regulation of light harvesting in green plants. Annu. Rev. Plant Physiol. *Plant Mol. Biol*;. **47:** 655–684.

**Horton P and Hague A.** (1988). Studies on the induction of chlorophyll fluorescence in isolated barley protoplasts: IV. Resolution of non-photochemical quenching. *Biochim Biophys Acta*; **932:** 107–115.

**Huesgen PF, Schuhmann H, Adamska I.** (2005). The family of Deg proteases in cyanobacteria and chloroplasts of higher plants. *Physiol Plant;* **123:**413–420.

**Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H.** (2006). Astaxanthin, a carotenoid with potential in human health and nutrition. *Journal of Natural Products*; **69**: 443–449.

**Jacob L and Zasloff M.** (1994). Potential therapeutic applications of magainins and other antimicrobial agents of animal origin: antimicrobial peptides. *Ciba Foundation Symposium*; **186**: 197-223.

**Jahns P, Depka B, Trebst A.** (2000). Xanthophyll cycle mutants from *Chlamydomonas reinhardtii* indicate a role for zeaxanthin in the D1 protein turnover. *Plant Physiology and Biochemistry*; **38:** 371-376.

**Jansen MAK, Mattoo AK, Edelman M.** (1999). D1–D2 protein degradation in the chloroplast: complex light saturation kinetics. *Eur J Biochem;* **260:**527–532.

**Jayraj J, Devlin R, Punja Z.** (2008). Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic research*; **17**: 489-501.

**Johanningmeier U and Fischer D.** (2009). Engineering chloroplasts of microalgae for the production of bioactive compounds. In: MT Giardi, G Rea and B Berra editors. Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors. LANDES BioScience and Springer, Publisher, in press.

**Johnson MP, Havaux M, Triantaphylides C, Ksas B, Pascal AA, Robert B, Davison PA, Ruban AV, Horton P.** (2007). Elevated zeaxanthin bound to oligomeric LHCII enhances the resistance of Arabidopsis to photooxidative stress by a lipid-protective, antioxidant mechanism. *The Journal of Biological Chemistry*; **282**: 22605–22618.

**Jones WHS** (Ed.) Hippocrates. Nutriment. 1923: 351.

**Kanazawa A and Kramer DM.** (2002). *In vivo* modulation of nonphotochemical exciton quenching (NPQ) by regulation of the chloroplast ATP synthase. *Proc Natl Acad Sci USA;* **99**:12789–12794.

**Kanervo E, Suorsa M, Aro EM.** (2005) Functional flexibility and acclimation of the thylakoid membrane. *Photochem Photobiol Sci;* **4(12):**1072-80.

**Kargul J and Barber JT.** (2008). Photosynthetic acclimation: structural reorganisation of light harvesting antenna–role of redox-dependent phosphorylation of major and minor chlorophyll a/b binding proteins. *FEBS Journal*; **275: 1056–1068.**

**Kindle KL.** (1987). Expression of a gene for a light-harvesting chlorophyll a/b-binding protein in *Chlamydomonas reinhardtii*: effects of light and acetate. *Plant molecular biology* **9**: 547–563.

**Kitajama M and Butler WL.** (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochima et Biophysica Acta*; **376:** 105–115.

**Knasmüller S, Nersesyan A, Misík M, Gerner C, Mikulits W, Ehrlich V, Hoelzl C, Szakmary A, Wagner KH.** (2008). Use of conventional and -omics based methods for health claims of dietary antioxidants: a critical overview. *Br J Nutr*; **99** E Suppl 1:ES3-52.

**Klein U, Salvador ML, Bogorad L.** (1994). Activity of the Chlamydomonas chloroplast *rbcL* gene promoter is enhanced by a remote sequence element. *Proceedings of the National Academy of Sciences of the United States of America*; **91**: 10819-10823.

**Klein U, De Camp JD, Bogorad L.** (1992). Two types of chloroplast gene promoters in *Chlamydomonas reinhardtii. Proceedings of the National Academy of Sciences of the United States of America*; **89**: 3453-3457.

**Korhonen H and Pihlanto A.** (2006). Bioactive peptides: production and functionality. *International Dairy Journal*; **16**: 945-960.

**Korhonen H and Pihlanto A.** (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current Pharmaceutical design*; **9**: 1297-1308.

**Kós PB, Deák Z, Cheregi O, Vass I.** (2008). Differential regulation of psbA and psbD gene expression, and the role of the different D1 protein copies in the cyanobacterium Thermosynechococcus elongatus BP-1. *Biochimica et Biophysica Acta;* **1777**: 74–83.

**Kota M, Daniell H, Varma S, Garczynski SF, Gould F, and Moar WJ.** (1999). Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proceedings of the National Academy of Sciences of the United States of America*; **96**: 1840-1845.

**Kruk J, Myśliwa-Kurdziel B, Jemioła-Rzemińska M, Strzałka K.** (2006). Fluorescence lifetimes study of α-tocopherol and biological prenylquinols in organic solvents and model membranes. *Photochem Photobiol*; **82:** 1309-1314.

**Külheim C, Agren J, Jansson S.** (2002). Rapid regulation of light harvesting and plant fitness in the field. *Science*; **297**: 91-93.

**Krzyzanowska J, Czubacka A, Oleszek W.** (2009). Dietary phytochemicals and human health. In: MT Giardi, G Rea and B Berra editors. Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors. LANDES BioScience and Springer, Publisher, in press.

**Ishikura K, Takaoka Y, Kato K, Sekine M, Yoshida K, Shinmyo A.** (1999). Expression of a foreign gene in *Chlamydomonas reinhardtii* chloroplast. *Jurnal of bioscience and bioengineering*; **87**: 307-314.

**Landrum JT and Bone RA.** (2001). Lutein, zeaxanthin, and the macular pigment. *Archives of biochemistry and biophysics*; **385**: 28-40.

**Lazar D.** (2006). The polyphasic chlorophyll *a* fluorescence rise measured under high intensity of exciting light. *Functionl Plant Biology*; **33**: 9–30.

**Lefebvre PA and Silflow CD.** (1999). Chlamydomonas: the cell and its genomes. *Genetics*;**151(1):**9-14.

**Lesgards J-F, Durand P, Lassarre M, Stocker P, Lesgards G, Lanteaume A, Prost M, Lehucher-Michel M-P.** (2002). Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environmental Health Perspectives*; **110:** 479-486.

**Lewin RA.** (1954). Mutants of *Chlamydomonas moewusii* with impaired motility. *J Gen Microbiol*; 11**:** 358-363.

**Lewin RA.** (1953). The genetics of *Chlamydomonas moewusii* Gerloff. *J Gen*; **51:** 543-560.

**Lewin RA.** (1952). Ultraviolet induced mutations in *Chlamydomonas moewusii* Gerloff. *J Gen Microbiol*; **6:** 233-248.

**Lewin RA.** (1951). Isolation of sexual strains of Chlamydomonas. *J Gen Microbiol*; **5:** 926-929.

**Lewin RA.** (1949). Genetics of Chlamydomonas-paving the way. *Bio Bull*; **97:** 243-244.

**Linden H.** (1999). Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. *Biochimica et Biophysica Acta;* **1446**: 203–12.

**Lohr M, Im CS, Grossman AR.** (2005). Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiology*; **138(1)**: 490-515.

**Lotan T and Hirschberg J.** (1995). Cloning and expression in *Escherichia coli* of the gene encoding beta-C-4-oxygenase, that converts beta-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. *FEMS Microbiology Letters;* **364**: 125–28.

**Luciński R and Jackowski G.** (2006). The structure, functions and degradation of pigment-binding proteins of photosystem II. *Acta biochimica polonica*; **53(4)**: 1-6.

**Luis P, Behnke K, Toepel J, Wilhelm C.** (2006). Parallel analysis of transcript levels and physiological key parameters allows the identification of stress phase gene markers in *Chlamydomonas reinhardtii* under copper excess. *Plant Cell Environment*; **29(11)**: 2043-54.

**Lutz KA, Knapp JE, Maliga P.** (2001). Expression of *bar* in the plastid genome confers herbicide resistance. *Plant physiology*; **125**: 1585-1590.

**Maccarrone M, Bari M, Gasperi V, Demmig-Adams B.** (2005). The photoreceptor protector zeaxanthin induces cell death in neuroblastoma cells. *Anticancer research*; **25**:3871-3876.

**Maliga P.** (2004). Plastid transformation in higher plants. *Annual review of plant biology*; **55**: 289-313.

**Manos J, Arthur J, Rose B, Bell S, Tingpej P, Hu H, Webb J, Kjelleberg S, Gorrell MD, Bye P, Harbour C.** (2009). Gene expression characteristics of a cystic fibrosis epidemic strain of Pseudomonas aeruginosa during biofilm and planktonic growth. *FEMS Microbiol Lett;* **292:** 107-114.

**Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, Weber A, McDonald TL, Mayfield SP.** (2007). Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast. *Plant Biotechnol J.;* **5(3):**402-12.

**Mares-Perlman JA, Millen AE, Ficek TL, Hankinson SE. (**2002). The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. Overview. *The Journal of nutrition*; **132**: 518S-524S.

**Maritim AC, Sanders RA, Watkins JB 3rd.** (2003). Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol*; **17:** 24-38.

**Mattson MP. Awareness of Hormesis.** (2008). Will Enhance Future Research in Basic and Applied Neuroscience. *Crit Rev Toxicol*; **38:** 633–639.

**Mattoo AK, Shukla V, Fatima T, Handa AK, Yachha SK.** (2009). Genetic engineering to enhance crop-based phytonutrients (nutraceuticals) to alleviate diet-related diseases. In: MT Giardi, G Rea and B Berra editors. Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors. LANDES BioScience and Springer, Publisher, in press.

**Mattoo AK and Edelman M.** (1987) Intramembrane translocation and posttranslational plamitoylation of the chloroplast 32-kDa herbicide- binding protein. *Proc Natl Acad Sci USA;* **84:**1497–1501.

**Mattoo AK, Hoffman-Falk H, Marder JB, Edelman M.** (1984). Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. *Proc Natl Acad Sci USA*; **81:**1380–1384.

**Maxwell DP, Laudenbach DE, Huner NPA.** (1995). Redox regulation of light-harvesting complex II and cab mRNA abundance in *Dunaliella salina*. *Plant Physiology* **109**: 787–795.

**Mayfield SP, Manuell AL, Chen S, Wu J, Tran M, Siefker D, Muto M, Marin-Navarro J.** (2007). *Chlamydomonas reinhardtii* chloroplasts as protein factories. *Current Opinion in Biotechnology*; **18(2)**: 126-33.

**Mayfield SP and Schultz J.** (2004). Development of a luciferase reporter gene, *luxCt*, for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal*; **37**: 449-458.

**Mayfield SP, Franklin SE, Lerner RA.** (2003). Expression and assembly of fully active antibody in algae. *Proceedings of the National Academy of Sciences of the United States of America*; **100**: 438-442.

**Mayfield SP, Yohn, Cohen A, Danon A.** (1995). Regulation of chloroplast gene expression. *Annual Review of Plant Physiology and Plant Molecular Biology;* **46**: 147-166.

**McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P.** (1995). Amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Bio/Technology*; **13**: 362-365.

**McCarthy SS, Kobayashi MC, Niyogi KK.** (2004). White Mutants of *Chlamydomonas reinhardtii* are defective in phytoene synthase. *Genetics*; **168(3)**:1249-57.

**Melis A, Neidhardt J, Benemann JR.** (1999). *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. *Journal of Applied Phycology*; **10**: 515-525.

**Merchant SS, Prochnik SE, Vallon O et al.** (2007). The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science;* **12**; **318**: 245-50.

**Messina MJ, Persky V, Setchell KD, Barnes S.** (1994). Soy intake and cancer risk: a review of the in vitro and in vivo data. *Nutrition and Cancer;* **21**: 113-131.

**Milanowska J and Gruszecki WI.** (2005) Heat-induced and light-induced isomerization of the xanthophyll pigment zeaxanthin. *J Photochem Photobiol B.* 1;**80(3):**178-86.

**Minagawa J and Takahashi Y.** (2004). Structure, function and assembly of photosystem II and its light-harvesting proteins. *Photosynthesis Research;* **82:** 241-263.

**Minagawaa J, Kramer DM, Kanazawa A, Crofts AR.** (1996). Donor-side photoinhibition in photosystem II from *Chlamydomonas reinhardtii* upon mutation of tyrosine-Z in the Dl polypeptide to phenylalanine. *FEBS Letters;* **389**: 199-202.

**Minagawa J, Kramer DM, Kanazawa A, Crofts A.** (1996). Donor-side photoinhibition in photosystem II from *Chlamydomonas reinhardtii* upon mutation of tyrosine-Z in the D1 polypeptide to phenylalanine. *FEBS letters;* **389:** 199-202.

**Minko I, Holloway SP, Nikaido S, Carter M, Odom OW, Johnson CH, Herrin DL.** (1999). *Renilla luciferase* as a vital reporter for chloroplast gene expression in Chlamydomonas. *Molecular & General Genetics*; **262**: 421-425.

**Mitchell P.** (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**: 144–148.

**Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D.** (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *The Plant Journal*; Epub ahead of print

**Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M, Shikanai T.** (2002). PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. *Cell*; **110(3):**361-71.

**Munekage Y, Takeda S, Endo T, Jahns P, Hashimoto T, Shikanai T.** (2001). Cytochrome b(6)f mutation specifically affects thermal dissipation of absorbed light energy in Arabidopsis. *Plant J.;* **28(3):**351-9.

**Müller K, Carpenter KL, Challis IR, Skepper JN, Arends MJ.** (2002). Carotenoids induce apoptosis in the T-lymphoblast cell line Jurkat E6.1. *Free radical research*; **36**: 791-802.

**Müller P, Li XP, Niyogi KK.** (2001). Non-photochemical quenching. a response to excess light energy. *Plant Physiol*; **125:** 1558-1566.

**Murata N, Takahashi S, Nishiyama Y, Allakhverdiev SI.** (2007). Photoinhibition of photosystem II under environmental stress. *Biochim Biophys Acta;* 1**767:**414–421.

**Newell CA.** (2000). Plant transformation technology: developments and applications. *Molecular Biotechnolgy*; **16**: 53-65.

**Newman SM, Gillham NW, Harris EH, Johnson AM, Boynton JE.** (1991). Targeted disruption of chloroplast genes in *Chlamydomonas reinhardtii*. *Molecular & general genetics*; **230(1-2)**: 65-74.

**Niedzwiedzki D, Krupa Z, Gruszecki WI.** (2005) Temperature-induced isomerization of violaxanthin in organic solvents and in light-harvesting complex II. J *Photochem Photobiol B.* 1;**78**(**2**):109-14.

**Niyogi KK.** (2000). Safety valves for photosynthesis. *Current opinion in plant biology*; **3**: 455-460.

**Niyogi KK.** (1999). Photoprotection revisited: genetic and molecular approaches. *Annual Reviewed of Plant Physiology and Plant Molecular Biology;* **50**: 333–59

**Niyogi KK, Bjorkman O, Grossman AR.** (1997). Chlamydomonas xanthophyll cycle mutants identified by video imaging of chlorophyll fluorescence quenching. *Plant Cell;* **9**: 1369–80.

**Niyogi K.K., Björkman O. and Grossman, A.R.** (1997b). The roles of specific xanthophylls in photoprotection. *Proc. Natl. Acad. Sci. USA* **94;** 14162–14167.

**Norris SR, Barrette TR, DellaPenna D.** (1995). Genetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation. *The Plant Cell*; **7**: 2139–49.

**Oksman-Caldentey KM and Arroo R.** (2000). Regulation of tropane alkaloid metabolism in plants and plant cell cultures. In: Verpoorte R, Alfermann AW, editors. Metabolic Engineering of Plant Secondary Metabolism. Dortrecht, The Netherlands; Kluwer Academic Publishers, p.253-281.

**Osyczka A, Moser CC, Dutton PL.** (2005) Fixing the Q cycle. *Trends Biochemestry Science*; **30(4):**176-82.

**Pfannschmidt T, Nilsson A, Tullberg A, Link G, Allen JF.** (1999). Direct transcriptional control of the chloroplast genes *psb*A and *psa*AB adjusts photosynthesis to light energy distribution in plants. *IUBMB Life*; **48(3)**: 271-6.

**Pacher P and Szabo C.** (2008). Role of the peroxynitrite-poly(ADP-ribose) polymerase pathway in human disease. *Am J Pathol*; 173: 2-13.

**Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Y Wright S, Hinchliffe E, Adams JL, Silverstone AL,Drake R.** (2005). Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nature biotechnology*; **23**: 482-487.

**Palace VP, Khaper N, Qin Q, Singal PK.** (1999). Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. *Free Radical Biology & Medicine*; **26(5/6):** 746–761.

**Peterson RB and Havir EA**. (2000). A nonphotochemical-quenching-deficient mutant of Arabidopsis thaliana possessing normal pigment composition and xanthophyll-cycle activity. *Planta. Jan*; **210(2):**205-14.

**Powles SB.** (1984). Photoinhibition of photosynthesis induced by visible light. *Annu Rev Plant Physiol;* **35:**14–44.

**Prokop J, Abrman P, Seligson A`L, Sovak M.** (2006). Resveratrol and its glycon piceid are stable polyphenols. *J Med Food*; **9:** 11-4.

**Przibilla E, Heiss S, Johanningmeier U and Trebst A.** (1991). Site-specific mutagenesis of the D1 subunit of Photosystem II in wild type Chlamydomonas. *Plant Cell*; **3**: 169–174.

**Pulz O and Gross W.** (2004) Valuable products from biotechnology of microalgae. *Appl Microbiol Biotechnol.*; **65(6):**635-48.

**Qian H, Li J, Sun L, Chen W, Sheng GD, Liu W, Fu Z.** (2009). Combined effect of copper and cadmium on *Chlorella vulgaris* growth and photosynthesis-related gene transcription. *Aquatic Toxicology;* **94**: 56–61.

**Rao AV and Rao LG.** (2007). Carotenoids and human health. Invited review. *Pharmacological Research*; **55**: 207–216.

**Ravanello MP, Dangyang Ke, Alvarez J, Huang B, Shewmaker CK.** (2003). Coordinated expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. *Metabolic Engineering*; **5**: 255-263.

**Raven JA and Allen JF.** (2003). Genomics and chloroplast evolution: what did cyanobacteria do for plants?. *Genome biology*; **4(3)**: 209-213.

**Reddy L, Odhav B, Bhoola KD.** (2003). Natural products for cancer prevention: a global perspective. *Pharmacology & therapeutics* **99**: 1-13.

**Rochaix JD.** (1995). *Chlamydomonas reinhardtii* as the photosynthetic yeast. *Annual review of genetics*; **29**: 209-30.

**Rock CL.** (1997). Carotenoids: biology and treatment. *Pharmacology & therapeutics*; **75 (3)**: 185-197.

**Romani A, Vignolini P, Galardi C, Aroldi C, Vazzana C, Heimler D.** (2003). Polyphenolic content in different plant parts of soy cultivars grown under natural conditions. *J Agric Food Chem*; **51:** 5301-5306.

**Römer S and Fraser PD.** (2005). Recent advances in carotenoid biosynthesis, regulation and manipulation. *Planta*; **221(3)**: 305–8.

**Römer S, Lübeck J, Kauder F, Steiger S, Adomat C, Sandmann G.** (2002). Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metabolic engineering*; **4**: 263-272.

**Rossel JB, Wilson IW, Pogson BJ.** (2002). Global changes in gene expression in response to high light in Arabidopsis. *Plant Physiology*; **130**: 1109–1120.

**Ruban AV, Lee PJ, Wentworth M, Young AJ, Horton P.** (1999). Determination of the stoichiometry and strength of binding of xanthophylls to the Photosystem II light harvesting complexes. *J Biol Chem* **274**: 10458–10465.

**Ruf S, Karcher D, Bock R.** (2007). Determining the transgene containment level provided by chloroplast transformation. *Proceedings of the National Academy of Sciences of the United States of America*; **104**: 6998-7002.

**Rupprecht J.** (2009). From systems biology to fuel-*Chlamydomonas reinhardtii* as a model for a systems biology approach to improve biohydrogen production. *Journal of Biotechnology*; **142(1)**: 10-20.

**Sager R.** (1955). Inheritance in the green alga *Chlamydomonas reinhardtii*. *Genetics*; 4**0:** 476-489.

**Sager R.** (1954). Mendelian and non-Mendelian inheritance of streptomicyn resistance in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA*; **40:** 356-363.

**Sager R and Granick S.** (1954). Nutritional control of sexuality in *Chlamydomonas reinhardtii. J Gen Physiol*; **37:** 729-742.

**Sager R and Granick S.** (1953). Nutritional studies with *Chlamydomonas reinhardtii. Ann New York Acad Sci*; **56:** 831-838.

**Salvador ML, Suay L, Anthonisen IL, Klein U.** (2004). Changes in the 5'-untranslated region of the rbcL gene accelerate transcript degradation more than 50-fold in the chloroplast of *Chlamydomonas reinhardtii*. *Current Genetics*; **45**: 176-182.

**Sajilata MG, Singhal RS, Kamat MY.** (2008). The carotenoid pigment zeaxanthin – A review. *Comprehensive reviews in food science and food safety*; **7**: 29-49.

**Sandmann G.** (2002). Molecular evolution of carotenoid biosynthesis from bacteria to plants. *Physiologia Plantarum;* **116**: 431–40.

**Satoh K and Yamamoto Y.** (2007) The carboxyl-terminal processing of precursor D1 protein of the photosystem II reaction center. *Photosynth Res;* **94:**203–215.

**Satoh K.** (1993) Isolation and properties of the photosytem II reaction center. In Deisenhofer J. and Norris J.R. (Eds.), *The Photosynthetic Reaction Center*. Academic Press Inc, New York, USA, pp. 289-318.

**Schledz M, Al-Babili S, von Lintig J, Haubruck H, Rabbani S, Kleinig H, Beyer P.** (1996). Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. *The Plant Journal;* **10**: 781– 92.

**Seddon JM, Ajani UA, Sperduto RD et al.** (1994). Dietary carotenoids, vitamin A, vitamin C, and vitamin E, and advanced age-related macular degeneration. *Journal of the American Medical Association*; **272:**1413-1420.

**Seo T, Blaner WS, Deckelbaum RJ.** (2005). Omega-3 fatty acids: molecular approaches to optimal biological outcomes. *Current opinion in lipidology*; **16**: 11-18.

**Shan Lu and Li Li.** (2008). Carotenoid metabolism: biosynthesis, regulation, and beyond. *Journal of Integrative Plant Biology*; **50(7)**: 778–785.

**Sharma J, Panico M, Shipton CA, Nilsson F, Morris HR, Barber J.** (1997). Primary structure characterization of the photosystem II D1 and D2 subunits. *J Biol Chem;* **272:**33158–33166.

**Sheng J, D'Ovidio R, Mehdy MC.** (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. *The Plant Journal*; **1(3)**: 345-54.

**Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY.** (1999). Seed specific expression of phytoene synthase: increase in carotenoids and other metabolic effects. *The Plant journal*; **20**: 410- 412.

**Shikanai T, Müller-Moulé P, Munekage Y, Niyogi KK, Pilon M.** (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. *Plant Cell.;* **15(6):**1333-46.

**Shikanai T, Munekage Y, Shimizu K, Endo T, Hashimoto T.** (1999). Identification and characterization of *Arabidopsis* mutants with reduced quenching of chlorophyll fluorescence. *Plant Cell Physiol;* **40:** 1134–1142.

**Silflow CD, Stern D, Vallon O, Zhang Z.Guerin M, Huntley ME, Olaizola M.** (2003). Haematococcus astaxanthin: applications for human health and nutrition. *Trends in Biotechnology*; **21**: 210–216.

**Singh U and Jialal I.** (2006). Oxidative stress and atherosclerosis. *Pathophysiology*; **13:** 129-42.

**Smith GM and Regnery DC.** (1950). Inheritance of sexuality in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA*; **36:** 246-248.

**Smith GM.** (1946). The nature of sexuality in Chlamydomonas. *Amer J Bot*; **33:** 625-630.

**Song YG, Liu B, Wang LF, Li MH, Liu Y.** (2006). Damage to the oxygen-evolving complex by superoxide anion, hydrogen peroxide, and hydroxyl radical in photoinhibition of photosystem II. *Photosynth Res;* **90:**67–78.

**Sopory SK, Greenberg BM, Mehta RA, Edelman M, Mattoo AK.** (1990). Free radical scavengers inhibit light-dependent degradation of the 32 kDa photosystem II reaction center protein. *Z Naturforsch;* **45c:**412–417.

**Spolaore P, Joannis-Cassan C, Duran E, Isambert A.** (2006). Commercial applications of microalgae. *Journal of bioscience and bioengineering*; **101**: 87-96.

**Stahl W and Sies H.** (2005). Bioactivity and protective effects of natural carotenoids. *Biochimica et Biophysica Acta*; **1740**: 101–107.

**Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA.** (2000). High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature biotechnology*; **18**: 333-338.

**Steinbrenner J and Linden H.** (2003). Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. *Plant Molecular Biology;* **52**: 343–56.

**Steinbrenner J and Linden H.** (2001). Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. *Plant Physiology;* **125**: 810–17.

**Strasser RJ, Srivastava A, Tsimilli-Michael M.** (2000). The fluorescence transient as a tool to characterize and screen photosynthetic samples. Probing photosynthesis: mechanism, regulation & adaptation, *Eds P. Mohanty, Yunus and Pathre, Taylor & Francis, London;* **pp**: 443-480.

**Suay L, Salvador ML, Abesha E, Klein U.** (2005). Specific roles of 5' RNA secondary structures in stabilizing transcripts in chloroplasts. *Nucleic Acids Research*; **33**: 4754-4761.

**Sumantran VN, Zhang R, Lee DS, Wicha MS.** (2000). Differential regulation of apoptosis in normal *versus* transformed mammary epithelium by lutein and retinoid acid. *Cancer epidemiology, biomarkers & prevention*; **9**: 257-263.

**Summerfield TC, Toepel J, Sherman LA.** (2008). Low-oxygen induction of normally cryptic *psb*A genes in Cyanobacteria. *Biochemistry;* **47**: 12939–12941.

**Sun Z, Gantt E, Cunningham FX Jr.** (1996). Cloning and functional analysis of the *β*-carotene hydroxylase of *Arabidopsis thaliana*. *The Journal of Biological Chemistry;* **271**: 24349– 53.

**Surh YJ.** (2003). Cancer chemoprevention with dietary phytochemicals. *Nature reviews. Cancer*; **3**: 768-780.

**Szabò I, Bergantino E, Giacometti GM.** (2005). Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *Embo Reports;* **6**: 629-634.

**Takahashi T, Inoue-Kashino N, Ozawa S, Takahashi Y, Kashino Y, Satoh K.** (2009). Photosystem II complex in vivo is a monomer. *The Journal of Biological Chemistry*; **284**: 15598-15606.

**Tepe B.** (2008). Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia virgata* (Jacq), *Salvia staminea* (Montbret & Aucher ex Bentham) and *Salvia verbenaca* (L.) from Turkey. *Bioresour Technol*; **99:** 1584-1588.

**Thomson LR, Toyoda Y, Delori FC, Garnett KM, Wong ZY, Nichols CR, Cheng KM, Craft NE, Dorey CK.** (2002). **a)** Long term dietary supplementation with zeaxanthin reduces photoreceptor death in light-damaged Japanese quail. *Experimental eye research*; **75**: 529-542.

**Thomson LR, Toyoda Y, Langner A, Delori FC, Garnett KM, Craft N, Nichols CR, Cheng KM and Dorey CK.** (2002). **b)** Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. *Investigative ophthalmology & visual science*; **43**: 3538-3549.

**Tyystjärvi T, Tuominen I, Herranen M, Aro EM, Tyystjärvi E.** (2002). Action spectrum of *psb*A gene transcription is similar to that of photoinhibition in *Synechocystis sp*. PCC 6803. *FEBS Letters*; **516(1-3):**167-71.

**Tyystja¨rvi E and Aro EM.** (1996). The rate constant of photoinhibition, measured in lincomycin-treated leaves, is directly proportional to light intensity. *Proc Natl Acad Sci USA;* **93:**2213–2218.

**Vági E, Rapavi E, Hadolin M, Vásárhelyiné Perédi K, Balázs A, Blázovics A, Simándi B.** (2005). Phenolic and triterpenoid antioxidants from *Origanum majorana* L. herb and extracts obtained with different solvents. *J Agric Food Chem*; **53:** 17-21.

**Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M and Telser J.** (2007. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*; **39:** 44-84.

**Vass I, Styring S, Hundal T, Koivuniemi A, Aro EM, Andersson B.** (1992). Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced QA species promote chlorophyll triplet formation. *Proc Natl Acad Sci USA;* **89:**1408–1412.

**Vattem DA, Ghaedian R, Shetty K.** (2005). Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry. *Asia Pac J Clin Nutr*; **14:** 120-30.

**Vila M, Couso I, Leon R.** (2008). Carotenoid content in mutants of the chlorophyte *Chlamydomonas reinhardtii* with low expression levels of phytoene desaturase. *Process biochemistry;* **43(10)**: 1147-1152.

**Von Lintig J, Welsch R, Bonk M, Giuliano G, Batschauer A, Kleinig H.** (1997). Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana*. *The Plant Journal;* **12**: 625–34.

**Walker TL, Purton S, Becker DK, Collet C.** (2005). Microalgae as bioreactors. *Plant cell reports*; **24**: 629-41.

**Walker, D.** (1990). The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis, *Research Institute for Photosynthesis, University of Sheffield.*

**Ward NC and Croft KD.** (2006). Hypertension and oxidative stress. *Clinical and Experimental Pharmacology and Physiology*; **33:** 872-876.

**Walters RG.** (2005). Towards an understanding of photosynthetic acclimation. *Journal of experimental botany;* **56**: 435-447.

**Wetzel CM and Rodermel SR.** (1998). Regulation of phytoene desaturase expression is independent of leaf pigment content in *Arabidopsis thaliana. Plant Molecular Biology*; **37**: 1045– 1053.

**Wahle KWJ, Brown I, Rotondo D, Heys SD.** (2009). Plant phenolics in the prevention and treatment of cancer. In: MT Giardi, G Rea and B Berra editors. Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors. LANDES BioScience and Springer, Publisher, in press.

**Wilson KE and Huner NP.** (2000). The role of growth rate, redox-state of the plastoquinone pool and the trans-thylakoid delta pH in photoacclimation of *Chlorella vulgaris* to growth irradiance and temperature. *Planta;* **212**: 93–102.

**Woitsch S and Römer S.** (2003). Expression of xanthophyll biosynthetic genes during light-dependent chloroplast differentiation. *Plant Physiol.;* **132(3):**1508-17.

**Wollman FA.** (2001). State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. *The EMBO Journal*; **20(14)**: 3623-3630.

**Wrona M, Ròzanowska M, Sarna T.** (2004). Zeaxanthin in combination with ascorbic acid or alpha-tocopherol protects APRE-19 cells against photosensitized peroxidation of lipids. *Free radical biology & medicine*; **36**: 1094-1101.

**Wrona M, Korytowski W, Zanowska M R´O, Sarna T,** T**ruscott TG.** (2003). Cooperation of antioxidants in protection against photosensitized oxidation. *Free radical biology & medicine*; **35**: 1319-1329.

**Xiong J, Subramaniam S, Govindjee.** (1996). Modeling of the D1/D2 proteins and cofactors of the Photosystem II reaction center. Implications for herbicide and bicarbonate binding. *Protein Science*; **5:** 2054-2073.

**Yamamoto Y, Aminaka R, Yoshioka M, Khatoon M, Komayama K, Takenaka D, Yamashita A, Nijo N, Inagawa K, Morita N, Sasaki T, Yamamoto Y.** (2008). Quality control of photosystem II: impact of light and heat stresses. *Photosynthesis Research*; **98(1-3)**: 589-608**.**

**Yamamoto Y.** (2001). Quality control of photosystem II. *Plant Cell Physiol;* **42:**121–128.

**Yamamoto HY, Bugos RC, Hieber AD.** (1999). Biochemistry and molecular biology of the xanthophyll cycle. *Photochem Caroten*; **8**: 293–303.

**Yamamoto HY**. (1979). Biochemistry of the violaxanthin cycle in higher plants. *Pure and applied chemistry*; **51**: 639-648.

**Yamane Y, Kashino Y, Koike H, Satoh K.** (1998). Effects of high temperatures on the photosynthetic systems in spinach: oxygen-evolving activities, fluorescence characteristics and the denaturation process. *Photosynthesis Research*; **57**: 51-59.

**Yamamoto Y, Ford RC, Barber J.** (1981). Relationship between thylakoid membrane fluidity and the functioning of pea chloroplasts. *Plant Physiol;* **67:**1069–1072.

**Yamamoto HY, Nakayama TOM, Chichester CO.** (1962). Studies on the light and dark interconversions of leaf xanthophylls. *Arch Biochem;* **97:** 168–173.

**Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I.** (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. *Science*; **287**: 303-305.

**Yongyue S, Shufen L, Can Q.** (2005). Solubility of ferulic acid and tetramethylpyrazine in supercritical carbon dioxide. *J Chem Eng Data*; **50:** 1125-1128**.**

**Youdim KA, Spencer JP, Schroeter H, Rice-Evans C.** (2002). Dietary flavonoids as potential neuroprotectants. *Biological chemistry*; **383**: 503-519.

**Zasloff M.** (2002). Antimicrobial peptides of multicellular organisms. *Nature*; **415(6870)**: 389-95.

**Zhang JJ, Ying J, Chang SH, Li B, Li ZS.** (2003). Cloning and expression analysis of violaxanthin de-epoxidase (*vde*) cDNA in wheat. *Acta Botanica Sinica;* **45**: 981–85.

*My greatest thanks go to my supervisors*

*Dr. Giuseppina Rea and Dr. Maria Teresa Giardi.*

*My family, for their encouragement and support, particularly at several turning points when I needed it.*

*My equal thanks go to all the colleagues of Council National of the Research for their knowledge and advice if I need.*

*Amina.*