Executive summary:

Culturing algal cells in photobioreactors requires high-density cells at high light intensities in order to get the maximum photosynthesis efficiency and biomass productivity. However, whereas photosynthesis efficiency is directly proportional to photon flux in low light, it rapidly decreases in high light intensities and excess light energy is dissipated to avoid oxidative damage. Several mechanisms exist to dissipate excess light energy, including the use a variety of exciton traps, which are collectively referred as non-photochemical quenchers (NPQ).

The SUNBIOPATH consortium investigated the high light response of algal cells (Chlamydomonas) and established a comprehensive list of genes and photosynthetic mechanisms that are crucial for growth in these conditions.

The consortium demonstrated that LhcSR3, a light harvesting protein, is able to sense the luminal pH of the chloroplast and to act as a quencher of excitation energy, the correlation between LhcSR3 and NPQ being clearly shown. This strict relationship demonstrated that LhcSR3 is a key protein involved in biomass production. The consortium also showed that induction of LhcSR3 by high light is depending on a Ca++ sensor protein (CAS), an important finding, which opens new insights into the regulation of the high light response of Chlamydomonas.

In parallel, the size of the light harvesting complex (Lhc) antenna was engineered with the aim of improving growth in photobioreactors. It is indeed thought that a smaller light-harvesting antenna size would increase light penetrance in deep layers of photobioreactors and reduce Lhc-dependent heat dissipation of absorbed light energy, thereby increasing photosynthetic efficiency in high light and high cell density. The SUNBIOPATH project demonstrated that it is possible to double photoconversion efficiency and productivity at high light (500 µE/m2/s) and high cell densities by reducing the size of the light harvesting antenna in the mutants isolated and characterized by the consortium. To bring these advantages into action, a wave-surface-reactor was developed, which maximizes the growth of the antenna-reduced mutants. Techno economic analysis revealed a reduction of the CO2 footprint for algal biomass production by about 30% using these mutants.

Besides the light harvesting complexes whose primary role is to collect light energy, efficient high light growth also depends on the subsequent reactions of the Calvin cycle for CO2 fixation. The consortium focused on sedoheptulose-1,7-bisphosphatase (SBPase) and Chlamydomonas mutant lines expressing a synthetic codon-optimized SBPase gene from

Dunaliella in the chloroplast were isolated. Some of them were found to have elevated biomass and higher starch levels.

Efficient photosynthesis relies on mitochondrial respiration, since excess reducing equivalents accumulating in the plastid can be exported and consumed by mitorespiration acting as a valve system. The consortium demonstrated that mTERF proteins, which are key regulators of mitochondrial expression, play a role in high light acclimation, by 'preparing' mitochondria for its function as electron sink under conditions where reducing equivalents accumulate in the plastid.

Production of high value products in the chloroplast is a challenge. The consortium identified several regulators of chloroplast gene expression that potentially hamper transgene expression. For efficient production of high value products, it is often required to express more than one transgene. Efficient co-transcription and co-translation of operons were demonstrated with certain specific combinations of genes, indicating that it is indeed possible to direct multiple transgene expression in the chloroplast of Chlamydomonas.

Project Context and Objectives:

SUNBIOPATH - towards a better sunlight to biomass conversion efficiency in microalgae - was an integrated program of research aimed at improving biomass yields and valorization of biomass for two Chlorophycean photosynthetic algae, Chlamydomonas reinhardtii and Dunaliella salina. For that purpose, the scientific workpackages were organized in order to get step by step insight into how algal cells use light energy, beginning with the primary reactions of photosynthesis and going deeper into the biochemistry of the cell to identify key components for biomass productivity. Then the general knowledge coming from these researches would be used to engineer cells for efficient transgenic expression and biomass production. In parallel, optimization of algal culture in photobioreactors was performed.

The linear photosynthetic electron transfer chain is composed of well known enzymes that use light to make the basic photochemistry involving extraction of electrons from water and transfer to NADP+, to generate NADPH. A proton-gradient is built up during electron transport and used by an ATP-synthase to generate ATP. Both NADPH and ATP are needed for CO2 fixation in the Calvin cycle for carbon compound synthesis. Coupled with the linear transfer of electrons, the auxiliary routes of electron flow (cyclic and chlororespiration), and changes in energy distribution between the two photosystems (state transitions) participate in the adaptation of photosynthesis to contrasting conditions. Another layer of control is performed by the antennas (made of pigments and proteins of the light harvesting complexes or Lhc) which not only collect energy and play a role in state transition but also dissipate energy under the form of heat or fluorescence in conditions of excess of light. For more efficient algal growth, it is important to understand the role of these alternative routes and discover ways for controlling energy dissipation.

Furthermore, efficient photosynthesis relies on mitochondrial respiration in the light. The rates of ATP and NADPH generation by the photosynthetic light reaction differ greatly from the rates of consumption by downstream pathways such as the Calvin cycle under certain conditions, so that accumulation of excess reducing equivalents in the plastid has to be prevented by valve systems. In order to ensure that photosynthesis is not perturbed by these imbalances, several strategies were evolved by photosynthetic cells that dissipate excess reducing power via the export of reducing equivalents which can be eventually used by the mitochondrial respiratory chain. Photosynthesis and photosynthetic acclimation are therefore dependent on a functional mitochondrial respiratory chain.

Lastly, the ability to manipulate chloroplast genes of Chlamydomonas represents an important molecular tool for dissecting and manipulating the process of photosynthesis and its regulation. Furthermore, the insertion of genes for novel enzymes provides the opportunity to alter chloroplast metabolism and increase the synthesis of high-energy fuel

molecules or high value molecules. However, manipulation of the chloroplast is still at in infancy because of a tight system of expression regulation.

Our main objectives were

(1) to improve algal growth in photobioreactors and identify key components for production of biomass,

(2) to manipulate chloroplast gene expression with the aim of producing biologicals,

(3) to optimize and establish models for growth in photobioreactors.

Objective 1: Improving algal growth in photobioreactors and identifying key components for production of biomass

a) Improving algal growth in high light

The productivity of phototrophic cultivation is highly dependent on the quantum yield of photosynthesis, the process by which primarily carbon (CO_2) is assimilated into organic matter. Whereas photosynthesis efficiency is directly proportional to photon flux in low light, it rapidly decreases in high light intensities and excess light energy is dissipated to avoid oxidative damage. However, maximum photosynthesis efficiency in high light would be desirable for culturing the algal cells, in order to get higher cell concentrations and higher biomass yields. Several mechanisms exist to dissipate excess light energy, among which are the use of electron sinks or a variety of exciton traps, which are collectively referred as nonphotochemical quenchers (NPQ). The consortium focused its researches on both of these ways of energy dissipation. Special attention was given to the role of the different proteins from the light harvesting complexes from Photosystems I and II during acclimation to different light intensities.

The consortium demonstrated that LhcSR3, a light harvesting protein, is able to sense the luminal pH of the chloroplast and to act as a quencher of excitation energy, the correlation between LhcSR3 and NPQ being clearly shown. This strict relationship demonstrated that LhcSR3 is a key protein involved in biomass production. The consortium also showed that induction of LhcSR3 by high light is depending on a Ca++ sensor protein (CAS), an important finding, which opens new insights into the regulation of the high light response of Chlamydomonas. To get a complete view of light harvesting efficiency, the roles of the other light harvesting proteins (Lhc) of the antenna in light capture and energy transfer to photosystem reaction centers as well as in NPQ have also been established by selectively inactivating the expression of the corresponding genes.

In parallel, the manipulation of the size of the light harvesting complex antenna has been the subject of a lot of attention with the aim of improving growth in photobioreactors. It is indeed thought that a smaller light-harvesting antenna size would increase light penetrance in deep layers of photobioreactors and reduce Lhc-dependent heat dissipation of absorbed light energy, thereby increasing photosynthetic efficiency in high light and high cell density.

To this aim, a library of mutants was built and screened with a computer-assisted micro-spectrophotometer device, designed by partners of the consortium. 10 mutants with lower chlorophyll content were isolated. Mutations responsible for this phenotype were identified and concern either chloroplast biogenesis, insertion of the light harvesting complexes in the thylakoid membranes or chlorophyll biosynthesis. These mutants were tested for growth first in small-scale photobioreactors (500 mL to 1.5 L) and then in large-scale photobioreactors (25 L). Three mutants presented higher photoconversion efficiency than the corresponding wild type in high light (500 µE/m2/s), with one of the mutants even doubling it (from 4% in wild type to 8% in the mutant). That corresponded to a productivity of 0.6 g/L.Day for the wild type and 1.2 g /L.Day for the mutant respectively. Simulation calculation suggested that this increase productivity could be the result of both less quenching at high light intensities due to lower chlorophyll content and less high light saturation.

Besides the light harvesting complexes whose primary role is to collect light energy, cyclic electron flow is an important player in the control of chloroplast energy and redox metabolism. One of the objectives of the consortium was thus to investigate cyclic electron flow by several means including genetic, proteomic and biophysical experiments. This allowed to demonstrate a Ca++-dependent regulation of cyclic electron flow, via the combined action of CAS, ANR1 and PGLR1, with ANR1 being important for the acclimation to high light, and thus crucial for efficient sunlight to biomass conversion.

In addition, efficient high light growth also depends on the subsequent reactions of the Calvin cycle for CO2 fixation. The aim of the consortium in that field was to find candidate rate-limiting enzymes in photosynthesis in order to manipulate them for increasing biomass. The consortium focused on sedoheptulose-1,7-bisphosphatase (SBPase). Chlamydomonas mutant lines expressing a synthetic codon-optimized SBPase gene from Dunaliella in the chloroplast were isolated. Several mutant lines that express the Dunaliella SBPase gene were found to have elevated biomass and higher starch levels.

Efficient photosynthesis relies on mitochondrial respiration, since excess reducing equivalents accumulating in the plastid can be exported and consumed by mitorespiration acting as a valve system. One of the aims of the consortium in that specific field was to find such actors and to demonstrate their role in high light growth. The consortium

demonstrated that mTERF proteins, which are key regulators of mitochondrial expression, play a role in high light acclimation, by 'preparing' mitochondria for its function as electron sink under conditions where reducing equivalents accumulate in the plastid. Thus these proteins are crucial for efficient sun to biomass conversion in fluctuating conditions such as outdoors cultivation. Strains overexpressing one of the mTERF proteins (MOC1) have been constructed and will constitute valuable tools for photobioreactor growth.

b) Understanding algal growth in outdoor conditions

When cultivated in outdoors systems, algal cells are subject to dark periods during which they consume their carbon resources (starch) accumulated during the day. An additional exogenous carbon source such as acetate for Chlamydomonas can be added to the medium and used as substrate for respiration to boost growth during the night. One of the aims of the project was to study the role of mitochondria in biomass conversion and assimilation of acetate in the cell. The consortium demonstrated that the isocitrate lyase enzyme from the glyoxylate cycle is essential for acetate utilization and that its lack has manifold consequences at the proteome and metabolome levels.

The consortium also focused attention on the mechanisms that occur during transition from dark to light. The comprehension of these processes is indeed important to facilitate the transition and avoid oxidative stresses. The consortium demonstrated the crucial role of the Mehler reaction during a dark-light transition in aerobic condition. However, when cells encounter anaerobic condition (due to sustained respiration in the dark), the consortium demonstrated that state transitions and the induction of a chloroplast hydrogenase independently promote the activation of photosynthesis.

Objective 2. Manipulating chloroplast gene expression with the aim of producing biologicals

Production of high value products in the chloroplast is still at in infancy. Transgene expression in Chlamydomonas can be limited due to negative feedback regulation that is exerted by unassembled proteins on the translation of their own mRNA (control by epistasy of synthesis, CES). The consortium put a lot of effort to the discovery of new regulators that negatively control chloroplast gene expression. Additionally, transgene expression can also be limited by proteolytic degradation. One of the objectives in that field was thus to find and characterize proteases from the chloroplast and to test transgene expression in a protease deficient context. The consortium achieved these aims by characterizating 4 factors that controls chloroplast mRNA genes and one protease (ftsH).

For efficient production of biologicals, it is often required to express more than one transgene. Efforts were thus made to develop trans-operons that could be co-transcribed and co-translated from a single transcription unit. The consortium demonstrated that expression of operons is possible.

In addition, an essential requirement for any recombinant platform is that transgene expression can be tightly regulated such that production of recombinant metabolites that are toxic to the host can be tightly controlled. This allows the uncoupling of host growth from production whereby the biomass is produced first and then it is induced to produce metabolites. Three strategies were thus investigated to establish an inducible expression system but none could be as efficient as the one, which is actually in use in the laboratory of one of the partners (riboswitch technique).

Objective 3. Optimizing and modelling algal growth in photobioreactors

When culturing cells in photobioreactors, the determination of the different parameters like temperature, pH, medium composition, CO2 supply (concentration/ partial pressure) and light intensity is critical for efficient growth. The first aim of the consortium was thus to determine the process conditions and the possible ranges of control. Once the best conditions for growth would be established, then growth kinetics of the wild type and different mutants generated by the consortium, would be determined to calculate the photoconversion efficiencies and biomass productivities.

The consortium demonstrated that the antenna reduced mutants showed higher photoconversion efficiency at middle and high light intensities. To bring these advantages into action, a wave-surface-reactor was developed to maximize the growth of the mutants. The material costs for this photobioreactor could be cut by half, and cheaper materials could be used because of reduced hydrodynamic pressure. Techno economic analysis revealed a reduction of the CO2 footprint for algal biomass production by about 30% using these mutants.

In addition, as an improvement for growth, one objective in that field was also to build a dynamic model to predict the response of the microalgae to various light regimes. The consortium reached that objective and provided a model which a good basis to simulate growth in real photobioreactors, which are characterized by mixing induced light/dark cycles in the range of 1 to 25 Hz.

In order to maximize valorization of the whole biomass, the consortium also looked for the potential of several microalgae as alternate substrate for biogas production focusing on the

biorefinery concept. We concluded that selected algae species can be good substrates for biogas production and that anaerobic fermentation can seriously be considered as final step in future microalgae-based biorefinery concepts.

Project Results:

Main S and T results/foreground

Project objectives

The general aim of the project was to improve biomass yields and valorization of biomass for two Chlorophycean photosynthetic microalgae, Chlamydomonas reinhardtii and Dunaliella salina. For that purpose, the work was organized into 5 scientific workpackages (WP) and one management WP that will be described elsewhere The scientific WP were organized in order to get step by step insight into how algal cells use light energy, beginning with the primary reactions of photosynthesis (WP1 and WP2) and going deeper into the biochemistry of the cell (WP3, WP4). Then the general knowledge coming from these WP is used to engineer cells for efficient transgenic expression and biomass production (WP2, WP3, and WP4). In parallel, optimization of algal culture in photobioreactors has been performed (WP5).

Titles of WP

WP1: Photochemistry and light capture efficiency

WP2: Light harvesting complex engineering

WP3: Identification and functional analysis of novel limiting enzymatic steps for biomass production

WP4: Control of chloroplast gene expression and metabolic engineering of the chloroplast

WP5: Optimization and valorization of algal culture in photobioreactors

General introduction

Optimal performance of photosynthesis requires a perfect balance between reactions of light capture and conversion into reducing equivalents and metabolic reactions involved into the utilization of energy. During linear electron flow, electrons are extracted from water at the level of photosystem II (PSII) and transferred to photosystem I (PSI) via plastoquinone (PQ), the cytochrome b6f complex (cyt b6f) and plastocyanin (PC). PSI donates electrons to NADP+ via ferredoxin (PetF) and ferredoxin-NADP+ reductase (FNR). A trans-thylakoid proton-gradient is built up during the electron transport and used by an ATP-synthase

(ATPsynth) to generate ATP. Both NADPH and ATP are needed for CO_2 fixation by ribulose-bisphosphate carboxylase oxygenase (Rubisco) and triose-phosphate (Trio-P) generation by the Calvin cycle for carbon compound synthesis (starch, protein, lipids). Linear electron flow is associated with state I conditions, in which the light harvesting complexes (LHC) of PSII (LHCII) are associated with PSII. Electron transport can also be cyclic, generating a trans-thylakoid proton gradient and ATP. During this process, electrons are reinjected from PSI either to the plastoquinone pool via a NADH dehydrogenase (Ndh) or to cyt b6f via a supercomplex including PSI, cyt b6f, FNR and PGR5/PGRL1. In C. reinhardtii, cyclic electron transport is associated with state II (LHCII associated with PSI). PSI and PSII collect energy via their light harvesting complexes (LHCI and LHCII), comprising several antenna proteins (Lhc) and pigments (chlorophyll a, chlorophyll b and carotenoids). Photosynthetic antennae are devoted not only to light harvesting but also to photoprotection. For an applied point of view, it would interesting to obtain algae with diminished size of their antenna for allowing a better penetrance of light in the photobioreactors, without disturbing the photoprotective effect.

Non-photochemical reduction of PQ involves Ndh (the Nda2 protein). In association with a plastid plastoquinol terminal oxidase (PTOX), Nda2 would perform chlororespiration, which is a respiratory-like electron transport chain.

Together with mechanisms allowing dissipation of energy at the level of the antenna (qE), the auxiliary routes of electrons (cyclic and chlororespiration), and changes in energy distribution between the two photosystems (state transitions) participate in the adaptation of photosynthesis to contrasting conditions (light modifications, nutrient and CO_2 availability, excess of salts, etc.) and it is important to understand their role and discover ways of control for a more efficient algal growth.

Moreover, ATP is also produced in two other places, the cytosol via glycolysis and mitochondria via the Krebs cycle and respiration. Metabolic exchanges occur between these compartments, which also contributes to algal adaptation to changing environments.

WP1: Photochemistry and light capture efficiency

General objective. The productivity of phototrophic cultivation is highly dependent on the quantum yield of photosynthesis, the process by which primarily carbon, but also nitrogen and sulphur are assimilated into organic matter. In the first WP, we aimed to better understand the array of mechanisms that regulates the utilization of absorbed light by the photosynthetic apparatus as a function of incident photon flux or as a function of the cellular

energetic status. This knowledge is the base for biotechnological improvement of solar light energy conversion into chemical energy to be used for industrial energetic purposes.

Objectives and achievements

For the whole period, our objectives were to build a high-performance micro-spectrophotometer device for computer-assisted detection of mutant colonies (goal 1, D1-1); to use it to generate an insertional mutant library affected in photosynthetic traits (goal 2, D1-2); to isolate mutants affected in state transition (goal 3, D1-3); to analyse protein members of the light harvesting antenna (Lhc) family (goal 4, D1-4); and to investigate the response to changing light (goal 5, D1-5).

D1-1: Micro-spectrophotometer device for computer-assisted detection of mutant colonies

The unicellular green alga Chlamydomonas reinhardtii is suitable for random nuclear genetic transformation and the availability of nuclear genome sequence information (Merchant et al., 2007) makes it an organism of choice for both forward and reverse genetic studies. We developed a strategy to identify mutants induced by random insertion mutagenesis with reduction in pigment content by fluorescence measurement using video-imaging devices coupled with spectrophotometer-fluorimeter. A new imaging set up was devised by partner 4, which is particularly suited to assess in vivo photosynthetic activity. The system specifically measures time-resolved chlorophyll fluorescence in response to light. It is composed of a fast digital camera equipped with a wide-angle lens for the analysis of samples up to 10 x 10 cm, i.e. entire plants or petri dishes. Together with new image processing techniques, it has proven most useful to screen large numbers of unicellular algal mutant colonies to identify those with subtle changes in photosynthetic electron flow (Johnson et al., 2009).

D1-2: Library of insertion mutants affected in photosynthetic traits.

Among a library of 10,000 mutants in Chlamydomonas obtained by insertional mutagenesis by using a device as described in the D1-1 section, partner 5 identified three mutants severely affected in chlorophyll content, about 8%, 20% and 50% of the wild-type level, named gun4, as1 and as2 (Bonente et al., 2011). As described in WP2, as1 and as2 mutants proved affected in chloroplast biogenesis mechanisms, while gun4 is affected chlorophyll biosynthetic pathway, resulting in either cases in a reduction of pigmentation per cell. A

number of other insertional mutants potentially altered in pathways for recycling reducing power have been produced and analysed by partner 4, with the set up described above in D1-1. Among these were two mutants altered in the MRL1 gene controlling RbcL expression, two mutants altered in the biogenesis of the ATP synthase and two mutants in the PTOX2 gene (see below). In addition, a dozen of mutants altered in PSI were sent for further characterization to partner 8, and a dozen of mutants altered in PSII biogenesis were sent to a colleague working in Munich on these issues.

D1-3: Identification of novel genes involved in state transitions

The process of state transitions involves the dynamic allocation of the mobile part of the LHCII antenna to PSII or PSI. This allows an appropriate redox balance of the photosynthetic electron transfer chain in response to changes in light or in the metabolic demands of the alga. State II, where the LHCII antenna is phosphorylated and associates with PSI, favors cyclic electron flow and the production of ATP. Partner 4 identified two of the mutants mentioned in D1-2 as altered in state transitions because of an increased reduction level of the PQ pool in darkness due to a mutation of the PTOX2 gene (see D3-1). Partner 4 also analysed several mutants from partner 5 that were potentially state transition mutants but proved unaffected in this process. Partner 1 investigated prolonged anaerobiosis in Chlamydomonas that leads to the expression of enzymes belonging to various fermentative pathways. Among them, oxygen-sensitive hydrogenases (HydA1/2) catalyze the synthesis of molecular hydrogen from protons and reduced ferredoxin (by PSI) in the stroma. Based on chlorophyll fluorescence induction kinetics typical of hydrogenase-deficient mutants, partner 1 set up an in vivo fluorescence imaging screening protocol allowing the isolation of mutants impaired in hydrogenase expression or activity, or altered in related metabolic pathways required for energy production in anaerobiosis. Out of 3000 clones from a small-sized insertional mutant library, five mutants were isolated and the most affected one was analyzed and shown to be defective for the hydrogenase HydG assembly factor. Such a mutant was also partially impaired in its ability to perform state transition after prolonged exposure to anaerobiosis (Godaux et al., 2013). Partner 8 prepared artificial micro-RNA vectors to knock down the expression of the Pph1 phosphatase in Chlamydomonas orthologous to, PPH1/TAP38 which is required for efficient de-phosphorylation of LHCII in Arabidopsis. Using PAM chlorophyll fluorescence measurements and 77K fluorescence emission spectra some lines that exhibit a tendency to favor State II were identified, as expected if the phosphatase cannot efficiently counteract the activity of the Stt7 kinase responsible for LHCII phosphorylation upon a transition to state II. However the phenotype of these lines tends to decrease over time, suggesting that the amiRNA constructs are themselves being silenced.

D1-4: Purified Light-harvesting antenna apoproteins

Various members of the Light harvesting proteins, Lhca and Lhcb, were produced in E.coli by partner 5 and reconstituted in vitro by adding specific pigments. In the case of antenna proteins of PSI, the Lhca proteins, their biochemical and spectroscopic characterization in vitro has been reported in (Mozzo et al., 2010). Pigment binding and spectroscopic properties were conserved among the nine Lhca subunits, with enrichment in red forms in Lhca2, Lhca4 and Lhca9. The main biochemical and spectroscopic properties of Lhca complexes showed good conservation through evolution from green algae to higher plants in contrast to Lhcb proteins that diverged more strongly. In particular recombinant Lhcbm1 and Lhcbm9 (which in vivo is expressed only under stress condition) have a lower fluorescence yield, with Lhcbm9 being particularly efficient in reducing singlet oxygen formation upon exposure to strong light. These results suggest that Lhcbm1 and 9 have a critical role in photoprotection of PSII. Additional studies were conducted by partner 5 with an Lhc-like protein from C. reinhardtii, named LhcSR3 that was previously identified after random mutagenesis as required for photoprotective thermal dissipation of light energy absorbed in excess in algae. Partner 5 demonstrated that LhcSR3 becomes protonated upon lumen acidification and that light energy absorbed by this protein is constitutively dissipated as heat, even if this process is enhanced upon protein protonation. LhcSR3 is thus able to sense the lumenal pH and to act as a quencher of excitation energy. Energy dissipation in LhcSR3 occurs through energy transfer to carotenoids and formation of a carotenoid radical cation that then rapidly undergoes charge recombination dissipating the excitation energy. This mechanism called CT quenching is common to Chlamydomonas and higher plants, even if located only on LhcSR3 proteins in the former, while located on monomeric Lhcb proteins in the latter and activated by the Lhc-like protein Psbs, which is the luminal pH sensor in higher plants (Bonente et al., 2011).

D1-5: Dynamics of light-energy dissipation in response to changing light intensities in Chlamydomonas

Absorption of light in excess of the capacity for photosynthetic electron transport is damaging to photosynthetic organisms. Several mechanisms exist to avoid photodamage, among which are the use of electron sinks or of a variety of exciton traps which are collectively referred to as nonphotochemical quenchers (Cardol et al., 2011). The latter are part of at least two major processes. State transitions (qT) represent changes in the relative antenna sizes of PSII and I. High energy quenching (qE) is the increased thermal dissipation of light energy triggered by lumen acidification. Partners1, 2 and 4 participated in a large international collaboration to investigate the respective roles of qE and qT in photoprotection. To this end a mutant (npq4 stt7-9) was generated in Chlamydomonas reinhardtii by crossing the state transition-deficient mutant (stt7-9) with a strain having a largely reduced qE capacity (npq4). The comparative phenotypic analysis of the wild type,

single mutants, and double mutants revealed that both state transitions and qE are induced by high light. Moreover, the double mutant exhibited an increased photosensitivity with respect to the single mutants and the wild type. Therefore, it appears that, besides qE, state transitions also play a photoprotective role during high light acclimation of the cells, most likely by decreasing hydrogen peroxide production (Allorent et al., 2013).

Partner 2 investigated the changes in PSII antenna size heterogeneity during state transitions: during a transition from state I to state II caused by plastoquinone reduction, mobile light-harvesting complexes are redistributed from PSII to PSI. This results in a decrease of the PSII antenna size. This change in PSII antenna size was estimated by deconvoluting the light-induced PSII fluorescence rise in the presence of DCMU, an inhibitor of electron transport beyond the primary PSII plastoquinone acceptor QA. This analysis was performed in Chlamydomonas during a transition from state II to state I upon admission of oxygen after a period of anaerobiosis that caused platoquinone reduction and establishment of state II. PSII heterogeneity in antenna sizes was observed in state I and in state II. Two PSII populations differed in their apparent antenna size by a factor of 2.5 and in their connectivity parameter. State transitions were adequately described as a change in the relative proportions of the two PSII populations with constant characteristics of antenna size and connectivity in the course of the process (de Marchin et al., submitted).

In another study, partner 2 demonstrated the prominent role of transient electron sinks during photosynthetic induction processes either through oxygen consumption by the Mehler reaction in aerobic conditions or by hydrogen production in anaerobic conditions. Indeed, a period of progressive induction of photosynthetic activity always occurs during the first minutes of adaptation to light after a period of darkness. During this process, enzymes of the Calvin cycle are activated by redox, pH and ATP-dependent processes. In Chlamydomonas and other microalgae, electron transport rates during induction in aerobic conditions are largely dependent on oxygen acting as a transient electron sink. This process was shown to depend neither on photorespiration nor on chlororespiration.

The absence of a significant dependence on chlororespiration was demonstrated using the PTOX2 knock-out mutant (Houille-Vernes et al., 2011). Thus a Mehler-type reaction at the reducing side of PSI is responsible for electron transport to oxygen during photosynthetic induction. In anaerobic conditions (due to sustained respiration in darkness), photosynthetic induction cannot rely on oxygen as transient electron sink. However, hydrogenase activity provides an alternative electron sink through electron transfer from ferredoxin to protons and formation of molecular hydrogen. The importance of hydrogenase in anaerobic photosynthetic induction was shown using a mutant deficient for hydrogenase activity. State transitions were also found to favour photosynthetic induction in such conditions, most likely by the increased rate of cyclic electron transport in state II. Therefore state transitions and the induction of a chloroplast hydrogenase independently promote the activation of

photosynthesis in anoxic conditions. A combination of defects in both mechanisms strongly compromised the ability of C. reinhardtii to resume photosynthesis in anoxic situations. We assume that the extensive state transition capacity of this alga together with its ability to dissipate electrons via hydrogenase-mediated H2 photo-evolution reflect the strong ability of Chlamydomonas to switch metabolism between anaerobic periods in the dark and aerobic periods in the light (Ghysels et al. 2013, Plos One accepted).

Oxygen also is a major electron sink in high light during steady-state photosynthesis. This conclusion arose from combined measurements by partner 2 of oxygen evolution and of electron transport rate by PAM-fluorimetry in cells cultivated photoautotrophically in photobioreactors under different light intensities in low or high CO2 conditions. As indicated by analyzing the behavior of a PTOX2-deficient mutant (Houille-Vernes et al., 2011), electron transport to oxygen occurs through Mehler-type reactions as also found during photosynthetic induction. Dissipation of excess excitation energy via electron transport to oxygen has to be considered as an important adaptation to CO2 limitation in high light, in combination with excitation energy dissipation in photosystems through energy-dependent non-photochemical quenching (NPQ). In Chlamydomonas, the latter does not occur if high light is not associated with CO2 limitation.

However high light also promotes antenna remodeling to accompany the dynamics of light-energy dissipation in response to changing light intensities in Chlamydomonas. This was shown by partner 5 who used Chlamydomonas reinhardtii cultures that have been grown at three different light conditions (35 - 60 - 400 µmol m-2s-1). Biochemical and physiological analysis demonstrated that acclimation to increasing light conditions lead to reduction of chlorophylls (Chls) and carotenoids (Cars) content per cell, with a decrease of Chl/Car ratio and increase of lutein and zeaxanthin content in HL on chlorophyll basis. Acclimation to high light conditions in higher plants is accompanied to a reduction of PSII antenna size and PSI/PSII ratio, while PSI antenna system is conserved: these data shade a different light on Chlamydomonas case, since little if any variations in PSII antenna system has been revealed by Western blot, pigment analysis and fluorescence kinetic measurements performed on cells infiltrated with DCMU. PSI antenna system on the contrary is characterized by a small but evident regulation on the quality of Lhca proteins bound to PSI core. HL condition also promoted the overexpression of LhcSR proteins, associated to heat dissipation in Chlamydomonas reinhardtii (Peers et al., 2009). Measuring heat dissipation of absorbed light energy in acclimated cells demonstrated that Non-photochemical quenching (NPQ) is strongly reduced in low or control light conditions, while high level of NPQ was detected in HL cells. This is in contrast with higher plants case, were LL adapted plants present a similar or even higher heat dissipation efficiency compared to HL plants: this discrepancy is likely due to the contrasting ecological niches of algae and higher plants, with the latter being exposed to sun-flecks that require efficient heat dissipation even in LL adapted plants.

WP2: Light harvesting complex engineering

General objective. Light harvesting complexes (LHC) comprise several polypeptides that bind photosynthetic pigments such as chlorophyll and carotenoids. They have a dual function in respect to photosynthesis: not only do they capture light and transfer energy to photosystem reaction centres but they also dissipate excess energy to avoid oxidative stress. This work package aims at the generation of microalgal strains displaying improved growth properties in photobioreactors. One strategy to achieve this goal is to engineer the light-harvesting antenna by genetic means yielding strains with size-reduced antenna systems at PSII. A smaller light-harvesting antenna increases light penetrance in deep layers of photobioreactors and reduces LHC-dependent heat dissipation of absorbed light energy thereby increasing photosynthetic efficiency.

Objectives and achievements

For the whole period, our objectives were to determine function of genes affecting light-harvesting process efficiency (goal 1, D2-1); to obtain mutants affected in the expression of the light-harvesting antenna proteins (goal 2, D2-2); to select mutant strains efficient for growth in photobioreactors with either low or high optical length (goal 3, D2-3).

D2-1: Function of genes affecting light-harvesting process efficiency

Biochemical and physiologic characterization of mutants screened in WP1 and described in D2-2, allowed to determine the functions of the different genes affected by mutation on light-harvesting process efficiency and chloroplast biogenesis. Silencing of Lhcb2+7 resulted in a clear decrease in the abundance of trimeric LHCII band in native green gel electrophoresis, implying that Lhcbm2+7 is an essential component of trimeric LHCII complex. Instead, small or even no decrease in LHCII trimers was observed in the npq5 mutant or in the mutants in which Lhcbm1 was silenced, implying that Lhcbm1, although a component of LHCII trimers, can be substituted by other gene products. Silencing of both LHCBM1 and LHCBM2+8 was efficient in decreasing the functional antenna size of PSII. Instead, while depletion of the former subunit decreased the capacity for excess energy dissipation but not the ability to perform state transitions, the opposite was true for LHCBM2+8, implying a specific role in the molecular mechanisms involved in these physiological mechanisms. Finally all genotypes here analyzed were efficient in photoprotection under high light conditions, implying that the major mechanisms of chlorophyll triplet quenching and reactive oxygen scavenging are shared by components of LHCII (Ferrante et al., 2012). The phenotypes of the mutants with multiple silencing Lhcbm4-

6-8 demonstrate that also Lhcbm4-6-8, tighter with Lhcbm1 are involved in induction of NPQ likely acting as partner of LhcSR3. Lhcbm3 and Lhcbm2-7 are instead involved in state transitions. The role of Lhcbm5 is still not clear due to inefficient silencing of the lhcbm5 gene. In collaboration partner 3 and 5 also characterized mutants silencing lhcbm9 gene, which is expressed only in stress condition, especially under sulfur deficiency. Our results demonstrate that Lhcbm9 is more efficient than other Lhcbm proteins in reducing ROS formation and its presence in PSII supercomplexes reduces fluorescence lifetimes, suggesting tis role as a quencher of excitation energy, increasing PSII photoprotection.

In parallel partner 5 characterized mutants obtained by insertional mutagenesis as described in WP1, characterized by having a reduced pigmentation. Analysis of photosynthetic polypeptides showed that in as1 and as2 mutants, Lhc subunits of both PSII and I are significantly reduced, although to different extents. In particular minor antenna protein CP26 and CP29 appear to be more affected than major Lhcb. While PSI core and ATP synthase are largely unaffected, PSII core subunits are less accumulated in the mutants. Since photosynthetic antennae are devoted not only to light harvesting but also to photoprotection, their reduction could lead to a phenotype more sensitive to photo-oxidative stress. Fortunately, this is not the case with both as1 and as2 that are not more photosensitive than wild type. Analysis of their growth performances confirms the hypothesis that a reduced optical density can indeed increase solar-to-biomass conversion efficiency in high light. Growth of mutants is improved in high light, while their reduced light-harvesting surface limits photosynthesis in low light. Differently, the wild type is unable to use all the applied irradiation in high light conditions and its growth kinetic is similar to that observed in low light as 2 displays a 40% increase in biomass yield in a self-shading test in multiple layers, simulating photobioreactor condition (see results on D2-3). Since PCR-based techniques to amplify the genomic sequence flanking the insertion in both as2 and as1 were unsuccessful, in contrast to the success obtained with gun4, partner 5 decided to sequence the genome through ILLUMINA® technology in order to identify the insertion site. In as2, only one insertion is present that perturbs different genes in the same locus. The best candidate mutated gene that could account for the observed phenotype is ftsY, encoding a component of the post-translational SRP-dependent pathway of the chloroplast (cpSRP), targeting Lhc proteins to thylakoids. In as1, the insertion in the arsA gene, homologous to prokaryotic arsA (arsenite transporter), correlates with the 'pale green' phenotype. arsA-like proteins have been studied so far in mammals and yeast, but not in plants, and they have been suggested to target C-tail-anchored (TA) proteins, characterized by a C-terminal signal peptide, to their target membranes. Components of the translocone complex of the outer membrane of the plastid (TOC) are TA proteins. Proteins involved in vesicle trafficking are TA members as well. Since certain Lhc proteins are inserted into the thylakoid membrane also in mutants for the cpSRP pathway, an alternative route could be engaged that could rely on vesicle trafficking. arsA could therefore be indirectly involved in the targeting of proteins to the chloroplast by regulating the biogenesis of TOC and/or vesicle trafficking. A mutated gene is responsible for the observed phenotype if introduction of a wild-type copy of this gene in the mutant is able to rescue the mutated phenotype. ARSA1 appears to be specifically involved in chloroplast function and its mutation did not alter the function of

other cellular compartments. As a matter of fact, the arsa1 mutation had a much stronger effect on photoautotrophy than on heterotrophy. Moreover, respiration rate was unaltered, suggesting maintenance of mitochondrial function. From the absence of any signal when assaying either whole cell extracts or membrane fractions using the antibody raised against Arabidopsis TOC34 in as1, we can hypothesize a role of ARSA1 in targeting and biogenesis of TOC34. Critical in this respect is the cellular location of ARSA1 that was carefully assessed by raising a polyclonal antibody in rabbit using highly purified recombinant ARSA1 expressed in E. coli that partner 5 used for detection in cell fractions. Recovery of ARSA1 in the cytosolic fraction rather than in the chloroplast fraction or thylakoid membranes ruled out the possibility that ARSA1 might resides in the chloroplast stroma to promote insertion of proteins into the thylakoid membrane.

These findings are highly significant and consistent with ARSA1 being needed for the TA protein TOC34 insertion in the outer chloroplast membranes. In turn, the absence of TOC34 would impair the import of nucleus-encoded photosynthetic proteins. While photosystem core complexes are encoded by the plastid genome, subunits of the light-harvesting antenna complexes (LHC) are encoded by the nucleus (LHCII, LHCB4-5 LHCA1-9) and then imported in the chloroplast where they are folded together with chlorophylls and carotenoids molecules. Impaired biogenesis of TOC in the present arsa1 mutant could explain the observed depletion of LHC polypeptides. In addition, import of enzymes involved in chlorophyll biosynthesis could be impaired (Reinbothe et al., 2005), limiting chlorophyll availability for chlorophyll-binding protein folding. The present as1 mutant has a pale green/yellow phenotype, still accumulates 8% of wild type chlorophyll level and is photosynthetically active, particularly in high light conditions. Interestingly, accumulation of the phosphoribulose kinase (PRK), a nucleus-encoded chloroplast protein involved in the Calvin-Benson cycle, is largely unaffected in the mutant. Similarly, the levels of some photosynthetic components, namely the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) as well as cytochrome b6f complex and ATP synthase, that comprise subunits encoded by both nuclear (Rubisco large chain) and chloroplast genes (AtpA, cytochrome f), are unaffected or reduced to a far lesser extent than the observed depletion in chlorophyll-binding polypeptides of photosystems, suggesting import of required nucleus-encoded assembling partners, despite the arsa1 mutation. These results suggest residual chloroplast protein import as the primary factor in determining the as1 phenotype that could derive from somehow lasting TOC activity catalyzed by TOC159, which is accumulated at similar level in cw15 and as1 (Formighieri et al., 2013). Another mutant screened in WP1 is a mutant that partner 5 selected for reduced pigmentation and identified to be affected in GUN4 accumulation. GUN4 is a regulatory subunit of Mg-chelatase involved in the control of tetrapyrrole synthesis in plants and cyanobacteria. Here, we report the first characterization of a gun4 insertion mutant of the unicellular green alga Chlamydomonas reinhardtii.

The mutant contains 50% of chlorophyll as compared to wild-type and accumulates ProtoIX. In contrast to the increase in LHC transcription, the accumulation of most LHC proteins is

drastically diminished, implying posttranscriptional down-regulation in the absence of transcriptional coordination. Partner 5 found that 803 genes change their expression level in gun4 as compared to wild-type, by RNA-Seq, and this wide-range effect on transcription is apparent under physiological conditions. Besides LHCs, partner 5 identified transcripts encoding enzymes of the tetrapyrrole pathway and factors involved in signal transduction, transcription, and chromatin remodeling. Moreover, perturbations in electron transport with a strongly decreased PSI-to-PSII ratio have been observed. This is accompanied by an enhanced activity of the plastid terminal oxidase (PTOX) that could have a physiological role in decreasing PSII excitation pressure (Formighieri et al., 2012).

Partner 3 had the task to investigate the translation control of light harvesting antenna by nucleus. The cytosolic translation repressor NAB1 has been previously identified in the green micro alga C. reinhardtii by screening a library of random insertion mutants with defects in light acclimation (Kruse et al., 1999). One of these mutants designated Stm3 was subjected to deeper analyses by partner 3 (Mussgnug et al., 2005), which revealed that the gene disrupted in Stm3 encodes the protein NAB1. Biochemical analyses demonstrated that NAB1 is a cytosolic translation repressor that sequestrates light-harvesting protein-encoding mRNAs in sub-polysomal mRNPs (messenger ribonucleoprotein complexes) separating such transcripts from the factors required for translation initiation. NAB1 harbours two cysteine residues Cys181 and Cys226 in its C-terminal RRM (RNA recognition motif) domain. Recent work demonstrated that the covalent modification of these cysteines is a means to regulate the translation repressor activity of NAB1 in vivo (Wobbe et al., 2009). Besides the C-terminal RRM domain NAB1 contains an N-terminal CSD domain and both domains are connected by a GAR (glycine-arginine rich) motif, which contains several RGG boxes known to be preferred arginine methylation sites of PRMT (protein arginine methyltransferases) enzymes(Bedford and Clarke, 2009). Arginines 90 and 92 of the GAR motif in NAB1 are dimethylated in vivo as determined by mass spectrometric analysis of immunopurified NAB1 (Blifernez et al., 2011). Inhibitor studies clearly demonstrated that arginine methylation is required to maintain NAB1 in an active state. Furthermore the extent of arginine methylation in vivo differed significantly between heterotrophic and phototrophic growth conditions with a high methylation state found after phototrophic cultivation where antenna size adjustment is vital. The methylation of arginines in the GAR motif of NAB1 therefore represents an important activity switch in the protein and controls thereby the translation of light-harvesting protein-encoding mRNAs in C. reinhardtii.

D2-2: Mutant strains affected in the expression of the light-harvesting antenna proteins

The antenna moiety of Photosystem II in Chlamydomonas reinhardtii is composed of LHCB4, LHCB5 proteins encoding the monomeric components CP29 and CP26 and by a number of LHCBM genes encoding the major LHCII complex. In order to gain knowledge on the structural organization of antenna system in green unicellular algae, an artificial microRNA

(amiRNA) silencing technology was used (Molnar et al., 2009), which allows discriminating between different genes and to coordinately silence genes sharing identical regions, while keeping unaltered the level of expression of other genes in the family. By screening mutants with increased Chl a/b ratio, partner 5 isolated mutants with silenced expression of genes Lhcbm1, Lhcbm2, Lhcbm3, Lhcbm5, Lhcbm6, Lhcbm8, and with multiple silencing Lhcbm2-7 and Lhcbm4-6-8. In addition, the npq5 mutant affected by insertion mutagenesis on Lhcbm1gene was analyzed (D2-1) (partner 5).

In parallel, partner 4 and 5 characterized mutant strains obtained by insertional or chemical mutagenesis affected on expression and accumulation of light harvesting proteins. Partner 4 identified six antenna mutant strains, four of which were isolated in their laboratory. Three types of antenna mutants were identified: a first group of three mutants turned out to be allelic, one of these mutants being known to bear a large deletion that affects, among other genes the chlorophyll oxygenase (CAO) gene responsible for the conversion of chlorophyll a into chlorophyll b. We identified a point mutation in the CAO gene of the two other mutants, which led to create a premature stop codon respectively in the first and second exon of the CAO gene. Therefore we have identified the first two specific chlb mutants altered exclusively in the CAO gene.

The resulting strains show a chla/chlb ratio above 15 (wild type has a chla/chlb ratio of 2.7), a total chlorophyll content highly light dependent, being close to the wild type content when grown in photoheterotrophic conditions under 20µE/m2/sec (ML conditions) but only 1/7th of it when grown under 5 µE/m2/sec (LL conditions). Their maximal fluorescence yield per cell is lower than that in the wild type, about 1/3rd in ML conditions down to 1/15th in LL conditions. The PSII antenna size, measured from the half time in the rise of the fluorescence induction curves with DCMU added is about 60% that of the wild type in ML growth conditions but five times lower when grown in LL conditions. These observations were consistent with the four times decrease in LHCII proteins in these mutants. Careful analysis of the major antenna protein subunits of LHCI showed that they were marginally affected with the exception of Lhca4 that was down to 20% of the wild type content in all light conditions. Another type of antenna mutant is specifically altered in LHCI but is also highly light sensitive. This is quite surprising because the two phenotypic traits should not be mechanistically linked. This mutant accumulates about 30% of LHCI antenna proteins in LL conditions, with a PSII antenna size slightly larger, by about 20% than the wild type. It shows no change in chla/chlb ratio but has only 30% of the wild type chlorophyll content per cell. The third type of antenna mutants shows a loss in all chla/b containing antenna proteins although chlb biosynthesis is not compromised. The two mutants belonging to that category are not allelic, therefore pointing to at least two genes that control this phenotype. We then sequenced several candidate genes that contribute to the biogenesis of chla/b binding proteins and found that these mutants harboured point mutations in the Alb3.1 and LTD genes respectively. The first mutation destroys the 5'splice site of the 5th intron of the Alb3.1 gene, whereas the other mutant carries a missense mutation that leads to a Q/R141

substitution in the LTD protein. In these two mutants, the chla/b ratio and chlorophyll content per cell are not light dependent, the former being above 4, the latter being 1/5th that in the wild type. The maximal fluorescence of these mutant is drastically decreased, being about 1/10th that in the wild type of a cell basis. The PSII antenna size is about 1/5th that in the wild type. It should be noted that only this third type of mutants shows a loss in the ability to perform state transitions, owing their deep alteration in LHCII biogenesis. Partner 5 instead identified three mutants severely affected in chlorophyll content, about 8%, 20% and 50% of the wild-type level, named gun4, as1 and as2 (Bonente et al., 2011). As described above, as1 and as2 mutants are affected on chloroplast biogenesis mechanisms, while gun4 is affected chlorophyll biosynthetic pathway, resulting in either case in a reduction of pigmentation per cell.

D2-3: Mutant strains efficient for growth in photobioreactors with either low or high optical length

Mutants as1 and as2 described in the previous section were tested for growing in photobioreactor (Task 2-3). At the beginning partner 5 tested the growth of these mutants in small-scale photobioreactor (400 ml) and in a home-made system in which different small flasks (20 ml) were shading each other, in order to artificially recreate the shading effect of different layer in a large scale photobioreactor. The results obtained demonstrate that mutant as2 is characterized by an increased growth in photobioreactor of 40% compared to WT, while the level of growth of WT and as1 were comparable. as1 and as2 mutant growth performances in large scale photobioreactors have been characterized in collaboration with partner 10: both mutants present an increase in photo conversion efficiency compared to WT from 4% to 5% and 6% respectively at 500 µE m-2s-1 both mutants present an increase in photo conversion efficiency compared to WT from 4% to 5% and 6% respectively at 500 µE m-2s-1 while the bf4 mutant isolated by partner 4 and affected in Abb3.1 presents a photoconversion efficiency of 8% at the same irradiance. That corresponds to a productivity of 0.6 g(l·d) for the wild type and 1.2 g/(l·d) for the bf4 mutant respectively. That was proven by simulation to be the result of less quenching at high light intensities due to correspondingly lower chlorophyll content (Jacobi et al. in preparation).

WP3: Identification and functional analysis of novel limiting enzymatic steps for biomass production

General objective. WP3 was dedicated to investigate in novel metabolic pathways and key enzyme components of energetic metabolism that are critical for biomass yield with micro algae. It was intended

-to modify the expression of proteins identified by comparing the proteome adaptation in cells of Dunaliella and Chlamydomonas metabolic mutants,

-to screen for new Chlamydomonas mutants affected in light-independent electron transfer pathways and

-to investigate in the function of specific enzymes known to play a key role either in the interplay between respiration and photosynthesis or to control respiratory activity.

The major project aim was to identify bottlenecks of photosynthesis in order to improve photosynthetic efficiency in unicellular green algal species. Research streams in WP3 had a specific focus on organelle interactions that influence the photosynthetic efficiency in the chloroplast knowing that in particular photosynthetic light acclimation relies on functional inter-organelle cross talk. We precisely investigated in aspects of mitochondria to chloroplast interactions that are known to play an important role in the functionality of the chloroplast as sunlight to chemical energy conversion system and on the interplay between nucleus, mitochondria and chloroplast to understand how the interaction between the respiratory and photosynthetic machinery mediates photosynthesis efficiency. Optimized mutants should be designed and their performance should have been tested in state of the art photobioreactors.

Objectives and achievements

For the whole period, our objectives were to identify key proteins involved in biomass production in Chlamydomonas and Dunaliella (goal 1, D3-1); to build vectors for silencing growth rate-limitating enzymes in Chlamydomonas and Dunaliella (goal 2, D3-2); to deliver a proteotypic peptide profiling platform available for further analysis of Chlamydomonas and Dunaliella mutant strains (goal 3, D3-3) ; to build vectors for overexpressing growth rate-limiting enzymes in Chlamydomonas and Dunaliella (goal 4, D3-4) ; to produce TILLING (Targeting Induced Local Lesions In Genomes) resources in Chlamydomonas and Dunaliella (goal 5, D3-5) ; to isolate Chlamydomonas and Dunaliella RNAi/miRNA mutants affected in metabolic processes (goal 6, D3-6) ; to obtain proteotypic peptide profiles of engineered mutant strains (goal 7, D3-7) ; to isolate Chlamydomonas and Dunaliella mutants with enhanced carbon assimilation rates and biomass accumulation in photobioreactors (goal 8, D3-8).

D3-1: Key proteins involved in biomass production in Chlamydomonas and Dunaliella

(a) Enzymes implicated in the utilization of acetate

Partner 1 identified several enzymes implicated in biomass production in Chlamydomonas when cells are grown in the presence of acetate either in the light (mixotrophic conditions) or in the dark (heterotrophic conditions) such as the Pdsw and nd4 subunits of the mitochondrial complex I of the respiratory chain (Barbieri et al., 2011; Larosa et al., 2012). Nda1 is a type II NADH dehydrogenase located on the inner side of the inner mitochondrial membrane. Double mutants affected in both complex I and Nda1 display severe decreased growth rate both in the light and in the dark, suggesting that Nda1 plays a critical role in the oxidation of matrix NADH in the absence of complex I (Lecler et al., 2012). Finally partner 1 characterized the isocitrate lyase enzyme (ICL1), which belongs to the glyoxylate cycle, as another important key element for efficient growth in the presence of acetate both in the light and in the dark (Plancke et al submitted). The Sco1 protein has been shown to be essential for cytochrome c oxidase assembly (complex IV of the respiratory chain) and for growth in the dark by partner 9.

(b) Enzymes implicated in the acclimation to high light and/or chlororespiration

Metabolic processes in chloroplasts and mitochondria are tightly linked by an intense inter-organellar crosstalk involving metabolite exchange. Efficient photosynthesis relies on mitochondrial respiration, since excess reducing equivalents accumulating in the plastid can be exported and consumed by mitorespiration acting as a valve system. Organellar gene expression (OGE) is among the candidate signal sources thought to initiate and modulate retrograde signalling events. Apart from an intense communication between organelles and nucleus the acclimation of photosynthetic eukaryotes to environmental changes critically depends on a tightly coordinated expression of the nuclear and organellar genomes. Partner 3 focused within WP3 his project work on mTERF proteins which act as key regulators of mitochondrial gene expression to obtain new insights into the complex interplay between chloroplast, mitochondria and nucleus. In vitro binding studies demonstrated that the key protein MOC1 acts as a transcription terminator in vivo (Wobbe and Nixon 2013, Nucleic Acids Res. in press). MOC1 accumulates under stress conditions such as heat shock or high light and inactivation of MOC1 results in a light-sensitive phenotype caused by a reduced non-photochemical quenching capacity (Nguyen et al., 2011). Partner 3 applied microarray studies to study the transcriptomic changes during high light acclimation in the MOC1 deletion mutant Stm6 and a MOC1-complemented strain. The data achieved highlight the crucial role of mTERF-regulated OGE within long-term light acclimation responses in photosynthetic eukaryotes which will help to improve phototrophic biomass productivity by establishing most efficient conditions for photon-to-biomass conversion (PCE) under fluctuating outdoor light conditions. The high light sensitive phenotype of stm6 indicates that MOC1 plays a role in preparing the mitochondrion for its function as an 'electron sink' under conditions where reducing equivalents accumulate in the plastid.

Partner 4 performed spectroscopic studies on photosynthetic electron transfer to monitor dark to light changes in the electron transfer chain and the impact of heterotrophic/mixotrophic acetate feeding on chloroplast carbon metabolism. The work was

based on a PTOX2 mutant, the major thylakoid-bound oxidase responsible for the oxidation of the plastoquinone pool in the dark through the chlororespiratory pathway, which was fully characterized at the functional level. As a result partner 4 confirmed the chloroplast localization of the upstream steps of the glycolytic pathway in Chlamydomonas and was able to show that cyclic electron flow around PSI is twice as fast in a starchless mutant fed with acetate than it is in the WT, and could describe properly the changes in the flux of electrons from carbohydrate metabolism that modulate the redox poise of the plastoquinone pool in the dark through chlororespiration.

Partner 5 considered the relationship between light use efficiency and Non-Photochemical-Quenching (NPQ) in the wild-type cells of Chlamydomonas reinhardtii and in some mutants, unable to induce NPQ, in different conditions of light intensity and light-dark pulse cycles. A photobioreactor exposed to light has an inherent light gradient due to light attenuation by the absorption of pigments in algae. Partner 5 measured a clear correlation between PCE (photoconversion efficiency) and Non-Photochemical-Quenching. They showed that the more stressful conditions produce higher NPQ and lower PCE. The characterization of growth in different conditions demonstrates that in Chlamydomonas reinhardtii LhcbSR3 proteins are induced in stress condition. When LhcSR is accumulated the thermal dissipation (NPQ) of light absorbed is highly induced. The correlation obtained between LhcSR3 and NPQ was clearly shown. This correlation supports the hypothesis that differences in NPQ level observed in acclimated cells depend on the level of LhcSR3 (Bonente et al., 2011).

Partner 6 performed comparative quantitative proteomics and genetic engineering of the chloroplast localized Ca2+ sensor protein (CAS), which revealed that the protein is required for light-dependent induction of LHCSR3, a protein essential for efficient energy dependent quenching of excess excitation energy (qE)(Petroutsos et al., 2011). This is an important finding and opens new insights into the regulation of the highlight (HL) response of C. reinhardtii. Full scans and MS/MS data sets of proteins were evaluated (Specht et al., 2011; Terashima et al., 2011) and led to the identification of ANR1 (anaerobic response 1) that is induced (1.7 fold) under anaerobic conditions (Terashima et al., 2010). Depletion of ANR1 by amiRNA expression showed that that the protein is required for effective acclimation to anaerobiosis and to high light (Terashima et al., 2012). Overall the data of partner 6 helped to establish a Ca2+-dependent regulation of cyclic electron flow (CEF) via the combined function of ANR1, CAS and PGRL1, associated with each other in a multi-protein complex (Terashima et al., 2012). These data shed new light on the regulation on CEF, which is crucial to photosynthesis because it participates in the control of chloroplast energy and redox metabolism and it is particularly induced under adverse environmental conditions. Thus the data provide new understanding of the control of photosynthetic electron transfer, a process that is crucial for efficient biomass production.

D3-2: Vectors for silencing growth rate-limitating enzymes in Chlamydomonas and Dunaliella

Several vectors have been constructed during the course of the project for silencing the expression of different genes: NDA1, PETO (encoding a phosphoprotein from cytb6f), CAS, ANR1, MOC1, LHCSR3, sedoheptulose-1,7-bisphosphatase (SBPase).

D3-3: Proteotypic peptide profiling platform available for further analysis of Chlamydomonas and Dunaliella mutant strains. Partner 6 developed a proteotypicprofiling platform and successfully analyzed protein dynamics in relation to biomass yield in wild-type that led to identification of CAS and ANR1 proteins (see D3-1). In addition, partner 6 employed quantitative proteomics to analyze photo-heterotrophic versus photo-autotrophic grown cells. These experiments allowed the quantitation of 1608 proteins and give detailed insights into how cell metabolism differs between the different trophic states (Hoehner et al., manuscript submitted). In collaboration with partner 1, an isocitrate lyase knockout mutant was analyzed by quantitative proteomics allowing in-depth characterization of metabolic remodeling due to ICL deficiency. In a new set of quantitative proteomics experiments partner 6 also analysed wild type, PGRL1 (Tolleter et al., 2011) and LHCSR3-deficient mutant (Peers et al., 2009) cells after shifting cultures from heterotrophic low light to highlight photo-autotrophic growth conditions. The quantitative mass spectrometric data are analysed by statistical clustering algorithms (Petroutsos et al., manuscript in preparation). In collaboration with partner 7, partner 6 established differential 15N/14N labelling for Dunaliella salina and investigated the response to high salt. These experiments allowed identification and quantification of Calvin cycle enzymes. Six Calvin cycle enzymes were found to be elevated at high salt in parallel with enhanced CO2 fixation rate, suggesting that they are candidate rate-limiting enzymes in photosynthesis.

D3-4: Vectors for overexpressing growth rate-limiting enzymes in Chlamydomonas and Dunaliella

Over-expression of MOC1 was performed to improve growth under conditions which are known to cause an accumulation of excess reducing equivalents (e.g. high-light, low CO2). Comparison between wild type strains and transformants revealed that the expression level of MOC1 cannot be further increased by employing the tested promoter systems and further indicated a high activity of the endogenous MOC1 promoter. These strains will represent valuable tools in future analyses aiming at the detailed characterization of MOC1 promoter regulation. In addition, two other vectors (gift of partner 3) for over-expression of the isocitrate lyase gene in Chlamydomonas have been constructed by partner 1. These vectors will be used for selection of transformants with better acetate utilization. Partner 7

constructed a synthetic codon-optimized sedoheptulose-1,7-bisphosphatase (SBPase) gene from Dunaliella tertiolecta for expression in the chloroplast of Chlamydomonas.

D3-5: TILLING (Targeting Induced Local Lesions In Genomes) resource in Chlamydomonas and Dunaliella

One of the roles of partner 5 was assessed as to construct a TILLING (Targeting Induced Local Lesions In Genomes) library in Chlamydomonas and Dunaliella (Task 3.5). This kind of resource has been previously applied to crop plants but not yet to algae. After the approval of project however, several problems arose with this technique, mainly due to difficulty in making reverse genetics in algae. Moreover being Chlamydomonas a haploid organism for most of its life cycle, the possibility to rescue mutants with mutations in essential genes is null. A similar tilling resource was produced in Berkeley by the lab of Kris Niyogi with rather limited success. In a meeting of the consortium in December 2010, it was suggested to change the method for obtaining a library of mutants in Chlamydomonas and to build a large insertional mutant library where each mutant could be identified by a Flanking Sequence Tag (FST). As a first step towards this goal, collaboration between partners 4 and 5 was launched under the supervision of Olivier Vallon (group member of partner 4) for completing a small-scale pilot of 96 mutants. The procedure successfully overcomes the major bottlenecks associated with this kind of project. To allow targeting even of essential genes, a vegetative diploid was used, where most mutations have no effect on growth, mating ability or storage. For long-term storage, the library was stored both in liquid nitrogen and as dormant, desiccation-resistant zygospores. Spores can be obtained by self-crossing, because the strains carry the iso1 mutation that allows differentiation of both mt+ and mt- gametes from the same stock. These tetraploid zygospores germinate and produce diploid progeny, ¼ of which are homozygous for the mutation. But because these strains also carry a constitutive transgenic GSM1 gene, they can differentiate into diploid 'pseudozygotes' that germinate to yield haploid progeny. Cells were transformed to paromomycin resistance, using a construct carrying AphVIII without a 3'-UTR. This leads to the production of a chimeric mRNA whose 3'-UTR is provided by the flanking Chlamydomonas genome. Symmetrically, the other end of the transforming DNA carries a strong promoter, but no 3'-UTR. Using 3'-RACE and Illumina sequencing of the pooled amplicon, FSTs from 68 out of 96 insertion sites were retrieved, some on both sides of the insertion. Out of 4 attempts, 3 mutants in genes of interest have been isolated and two have been brought to the haploid state (the third one is essential).

D3-6: Chlamydomonas and Dunaliella RNAi/miRNA mutants affected in metabolic processes.

Partner 1 has investigated Chlamydomonas mutants affected in enzymes responsible for growth on acetate. Respiratory-deficient mutants affected in different enzymes of the

respiratory chain have been analyzed in mixotrophic conditions. They all show decreased respiration and photosynthesis rates. In addition, whereas protein content does not vary, starch and neutral lipids contents per cell are strongly decreased. This suggests that the respiratory deficient mutants present reduced metabolic activities, responsible for decreased accumulation of carbon resources (Lecler et al., 2011). A knock out in the microalga Chlamydomonas affecting the isocitrate lyase gene (see D3-1) resulted in altered growth in the presence of acetate, reduced acetate assimilation, concomitant reduced respiration and a variety of other metabolic pathways, highlighting the central role of ICL for efficient growth.

Further RNAi-strains were constructed by partner 3 showing an up to 70% reduction in the mRNA level of the mitochondrial DNA binding protein that was shown to play an essential role in the regulation of sun to biomass (MOC1) (see D3-4). The phenotype of the knock-down strains reflects the phenotype observed for the corresponding knockout strain thus demonstrating again the crucial role of this protein efficient light to biomass conversion under increasing light conditions. Seven miRNA lines of Chlamydomonas with reduced mRNA expression levels of sedoheptulose-1,7-bisphosphatase (SBPase) were prepared by partner 7. All lines showed reduced CO2 fixation rates that paralleled reduced levels of SBPase activity, indicating that SBPase is a rate-limiting enzyme in photosynthesis in Chlamydomonas.

D3-7: Proteotypic peptide profiles of engineered mutant strains

In collaboration with partner 1, partner 6 analyzed an isocitrate-lyase knockout mutant by quantitative proteomics using differential 15N/14N metabolic labeling. Between 1684 and 1792 proteins have been identified in the four datasets, respectively and allowed in-depth characterization of metabolic remodeling due to ICL deficiency (Plancke et al., manuscript submitted). Comparative quantitative analysis of a CAS mutant strain unable to acclimate to high light revealed among 423 quantified proteins, 31 proteins that are repressed in the mutant versus the wild-type and might be important for the high-light response in C. reinhardtii. In a new set of quantitative proteomics experiments partner 6 analysed the highlight response of C. reinhardtii using differential 15N/14N metabolic labelling. They analysed wild type, PGRL 1 (Tolleter et al., 2011, 2011) and LHCSR3-deficient mutant (Peers et al., 2009) cells after shifting cultures from heterotrophic low light to highlight photo-autotrophic growth conditions. The quantitative mass spectrometric data are currently analysed by statistical clustering algorithms.

D3-8: Chlamydomonas and Dunaliella mutants with enhanced carbon assimilation rates and biomass accumulation in photobioreactors

The strict relationship between NPQ and productivity (see D3-1) and NPQ and LhcSR3, demonstrate that LhcSR is a key protein involved in biomass production in Chlamydomonas. Partner 5 tested this hypothesis by measuring PCE in npq4 mutant compared to WT and the light use efficiency and productivity in npq4 is 22% higher than WT at 200 µmol m-2s-1 under L/D cycles of 1s. Together with the increase PCE described for mutants with reduced pigmentation, this result finally suggests that the quality and quantity of Lhc and Lhc-like protein is crucial to determine biomass productivity in Chlamydomonas. Partner 10 has already started to deeply investigate into the influence of variations in different parameters like temperature, pH, medium composition, CO2 supply (concentration/ partial pressure) and light intensity in order to optimize the process conditions and possible ranges for control in the future. Partner 7 and partner 8 collaborated to prepare Chlamydomonas mutant lines expressing a synthetic codon-optimized SBPase gene from Dunaliella tertiolecta in the chloroplast. Several mutant lines that express the D. tertiolecta SBPase gene were found to have elevated biomass and higher starch levels.

WP4: Control of chloroplast gene expression and metabolic engineering of the chloroplast

The overall goal of this work package was to build on the existing expertise in chloroplast molecular-genetic to gain a fully understanding of the molecular mechanisms underlying the expression and regulation of chloroplast genes in the green alga Chlamydomonas. This knowledge would then allow the exploitation of the algal chloroplast as an expression platform in which transgenes are introduced into the chloroplast genome with a view to making novel proteins and metabolites. Below is a summary of the research work carried out by partners 4, 8 and 9.

Objectives and achievements

For the whole period, our objectives were to obtain an optimal inducible system for transgene expression in the chloroplast (goal 1, D4-1); to identify factors controlling the epistasy of synthesis (CES) process, which controls in turn the synthesis rate of key chloroplast proteins (goal 2, D4-2); to obtain a modular system in which multiple genes can be assembled together in an operon that is co-transcribed and co-translated (goal 3, D4-3); to isolate Chlamydomonas nuclear mutants affected in chloroplast proteases (goal 4, D4-4); to obtain Chlamydomonas strains with enhanced chloroplast transgene expression (goal 5, D4-5); to obtain Chlamydomonas strains expressing biosynthetic genes for C10 monoterpenoids (goal 6, D4-6).

D4-1: Optimal inducible system for transgene expression in the chloroplast

An essential requirement for any recombinant platform is that transgene expression can be tightly regulated such that production of recombinant metabolites that are toxic to the host can tightly controlled. This allows the uncoupling of host growth from production whereby the biomass is produced first and then it is induced to produce the metabolite. Several strategies were investigated.

i) Temperature-regulated control of transgene expression using the E. coli lac repressor (partner 8). An attempt was made to create an inducible system based on the expression of a temperature sensitive variant of the lac repressor in the chloroplast, coupled to a modified chloroplast promoter element containing binding sites for the E. coli lac repressor. In this system, expression of a gene-of-interest linked to the promoter would be prevented by repressor binding at the non-permissive temperature. However, a rise in temperature would result in a lack of binding and induction of expression. Earlier, we had shown the successful expression in the chloroplast of a synthetic gene encoding the lac repressor. However, our analysis of a modified petA promoter that contained two binding sites for the repressor showed that the modifications resulted in a non-functional promoter. We therefore designed and constructed a new promoter based on that from psaA and shown that this is functional in the chloroplast when linked to a test transgene encoding human growth hormone (hGH). When the transgene was introduced into a strain expressing the lac repressor, no hGH was detected at 25oC, whereas a control strain in which part of the repressor gene had been deleted showed normal hGH levels. Unfortunately, it was subsequently discovered that this accumulation of hGH (but not other recombinant proteins that were tested) is affected by temperature such that no protein is detectable at 35oC, even in the control strain. This negates the use of hGH as a test protein for our induction system. The transgenic lines are therefore being re-made using another test protein (CodA) that was shown to accumulate without problem at 35oC.

ii) Use of the E. coli FimE DNA recombinase as a molecular switch for induction of transgene expression (partner 8). The aim of this project was to express the FimE gene in the Chlamydomonas nucleus under the control of an inducible promoter (from CYC6) such that the FimE protein would be targeted into the chloroplast. Once in the chloroplast, the recombinase would recognise short target sequences flanking a promoter element and 'flip' its orientation, thereby turning on expression of a downstream transgene. However, repeated attempts to express in the nucleus a codon-optimised version of the E. coli recombinase protein FimE and target it into the algal chloroplast have proved unsuccessful. The failure to detect any FimE protein in over 100 transgenic lines, despite the fact that FimE was fused to various strong nuclear promoters, has prompted a redesign of the expression strategy. Partner 8 is currently building a new construct for nuclear transformation in which the FimE gene is translationally fused to a selectable marker (BLE) via a 2A peptide sequence. Such sequences are found in various viral polyproteins and mediate the failure of the 80S ribosome to form a peptide bond between a glycine and proline within the 2A

sequence. As a consequence, two separate proteins are made during the same translation event - and importantly, both proteins are synthesised to the same level. Such 2A sequences have been used effectively to synthesis multiple proteins in various model eukaryotes including C. reinhardtii (Rasala et al., 2012).

iii) Riboswitches to control transgenes (partner 9). To investigate the usefulness of bacterial riboswitches for the experimental manipulation of chloroplast gene expression, two riboswitches were tested. The first is the synthetic theophyllin-responsive 'on' riboswitch that was previously shown to function in tobacco chloroplasts (Verhounig et al., 2010). The second is a putative 'off' riboswitch from a cyanobacterial thiC gene. These riboswitches were placed between the psaA promoter /5'UTR and the lucCP reporter, and homoplasmic transformed lines were obtained. However with either riboswitch, luciferase activity was very low, was strongly dependent on the physiological state of the culture, and was not markedly responsive to theophyllin or thiamine respectively. These results indicate that the tested riboswitches would not be useful for controlled chloroplast gene expression in Chlamydomonas and that other inducible systems that were developed in Geneva perform better (Ramundo et al., 2013).

D4-2: Factors controlling the epistasy of synthesis (CES) process, which in turn controls the synthesis rate of key chloroplast proteins

i) Nuclear factors mediating CES (partners 4 and 9). Previous work in these partners labs had shown that transgene expression in the Chlamydomonas chloroplast can be limited due to the negative feedback regulation that is exerted by unassembled subunits on the translation of their own mRNA (control by epistasy of synthesis (CES); reviewed by (Choquet and Vallon, 2000). The expression of a transgene placed under the control of the psaA promoter and 5'UTR is enhanced in a nuclear mutant background that prevents trans-splicing of psaA and hence accumulation of the PsaA protein (Wostrikoff et al., 2004; Michelet et al., 2011). However with only one exception (Boulouis et al., 2011), the identity of the nuclear-encoded proteins that mediate this type of negative feedback regulation has not been elucidated. In an effort to identify such regulators three trans-acting factors were investigated; namely, Taa1, Mac1 and Mbb1.

The nuclear mutant taa1 is non-photosynthetic and PSI deficient. Because it cannot express a psaA:aadA reporter (introduced by chloroplast transformation) that should confer resistance to spectinomycin, but can express the control atpA:aadA, it is inferred to be defective in the expression of the chloroplast psaA gene. The taa1 mutant shows reduced levels of psaA mRNA, but fails to accumulate any PsaA protein, suggesting defects at the level of mRNA stability and translation. In the progeny of a cross of taa1 to a chloroplast reporter strain with psaA:aadA, resistance to spectinomycin segregates with the wild-type allele, indicating that the target of Taa1 is in the 5'UTR of psaAex1. The TAA1 gene encodes a

protein of 202 kDa which contains seven tandem repeats of the OPR family of helical repeat proteins and a RAP domain, and is thus predicted to bind RNA. A polyclonal antibody against Taa1 has recently been produced and conditions optimized for the detection of this technically challenging protein by immunoblotting. It was found that in different wild-type genetic backgrounds the amount of Taa1 protein is variable even though the PsaA protein accumulates to similar levels. However, preliminary results indicate that under conditions of iron limitation, when PSI and in particular PsaA are downregulated, there is a concomitant reduction in the amount of Taa1 protein.

In the mac1 mutant, the psaC mRNA fails to accumulate because of a defect in stability. We have identified the MAC1 gene, which encodes a protein of the TPR family. Our preliminary results indicate that the Mac1 protein may also be down-regulated under iron limitation. Mbb1 is required for the stable accumulation of psbB mRNA, and like Mac1 it is a member of the TPR family. While the TPR domains are well known as mediators of protein-protein interactions, there is growing evidence that members of a subfamily may be RNA-binding proteins (Hammani et al., 2012). Using immunoprecipitation followed by electro-mobility shift assays (EMSA), we evidence was obtained that Mbb1 associates with psbB mRNA.

ii) The role of the TDA1 factor (partner 4). The TDA1 nuclear gene controlling the expression of subunit α of the chloroplast ATP synthase in Chlamydomonas, which is critical for biomass production in phototrophic conditions, has been fully characterized. It is specifically required for translation of the chloroplast atpA transcript. The sequence of TDA1 contains eight copies of a degenerated 38 residues motif, or Octotrico Peptide Repeats (OPR), previously described in a few other trans-acting factors targeted to the Chlamydomonas chloroplast. Interestingly, a fraction of the untranslated atpA transcripts are sequestered into high-density, non-polysomic, ribonucleoprotein complexes.

The results suggest that TDA1 has a dual function:(i) trapping of a subset of untranslated atpA transcripts into non-polysomic complexes and(ii) translational activation of these transcripts. Its role in the CES process that controls expression of subunit α via a trans-activation by the β subunit can now be studied.

iii) The role of Mca1 in the CES process (partner 4). The role of the Mca1 factor as a translational enhancer for cytochrome f expression has been further documented by its action in strains altered for the CES process, i.e. the assembly-dependent regulation of cytochrome f translation. As a major conclusion drawn from these experiments, MCA1 turns out to be the nuclear trans-acting factor that controls the CES process for cytochrome f expression. Attempts to probe a direct molecular interaction between cytochrome f and MCA1 are currently being developed in two hybrid experiments.

iv) A genetic screen for the identification of mutants in the CES process (partner 4). An experimental strategy has been devised for isolating mutant strains escaping the CES process, which negatively down-regulates the expression of most chloroplast genes, thereby

preventing high accumulation of their products. As a first attempt, Chlamydomonas mutants with impaired auto-regulation of cytochrome f synthesis, the best characterised CES protein, were constructed. In strains defective for cytochrome b6f assembly, expression of the cytochrome f gene, petA and of 5'petA-driven reporters is reduced. In particular, the 5'petA-aadA chimera only confers resistance to low levels of aminoglycosides. By contrast mutant strains defective for the CES process show high expression of this chimera and resist high concentrations of the antibiotics, allowing the selection of the desired mutants. To avoid false positives unrelated to the CES process that correspond to spontaneous antibiotic resistance mutations, a second 5'petA-driven chimeric gene was introduced in the strain used for mutagenesis. The 5'petA-psbB chimera, encoding the CP47 subunit of the PSII complex, is poorly expressed when cyt. b6f assembly is impaired and the resulting strain exhibits the fluorescence signature of PSII mutants. In the desired mutants, the higher expression of this second reporter restored assembly of PSII and a fluorescence phenotype typical of cytochrome b6f deficiency, easily screened among the spontaneous antibiotic-resistant clones that retain the PSII-defective fluorescence signature.

After construction of the appropriate strain and mutagenesis, mutants showing high expression of both the 5'petA-aadA and 5'petA-psbB reporter genes were recovered based on their resistance to high levels of antibiotics and restored PSII expression, as deduced from their fluorescence pattern. It turned out that the loss of cytochrome f autoregulation in these strains originated from the mere deletion of the cytochrome f coding sequence, because of a recombination event between direct repeats flanking the petA gene. This first trial therefore, although it validated the chosen selection strategy, did not lead to the isolation of the desired mutants. To circumvent this flaw a new version of the strain to be subjected to mutagenesis has been constructed where these repeats have been removed and the petA gene relocated between two essential genes to prevent its deletion.

D4-3: Modular system in which multiple genes can be assembled together into a trans-operon that is co-transcribed and co-translated (partner 9).

The goal of this project was to develop a simple operon assembly system that allows the efficient translation of multiple transgenes in the chloroplast from a single transcription unit. The design strategy was based on the hypothesis that the overlap (or close association) of the stop codon of the upstream ORF with the start codon of the next ORF should facilitate 'translational coupling' where the termination of translation and its re-initiation at the next translation start are intermittently coupled. Evidence of such coupling is found in both plant and algal chloroplast genomes, as well as bacterial and viral systems. We therefore used a synthetic biology approach to create as series of two-ORF constructs in our chloroplast expression vector pASapI and introduce these into the Chlamydomonas chloroplast genome. The ORFs encode an endolysin (cpl1) and the kanamycin resistance gene aphA6 and were coupled in this order but in three different ways (a 5bp gap between the TAA and ATG; a 1bp

gap; a 1bp overlap). Transformant lines were obtained for all three. In addition, equivalent transformant lines were created in which translation of the upstream cpl1 was blocked by the introduction of a TAA stop codon within the coding region.

Expression of both transgenes was observed (as determined by western analysis for Cpl1 and kanamycin resistance for AphA6), although expression of aphA6 via translational coupling was less efficient than that of the gene expressed on its own from the same atpA promoter/5'UTR. Furthermore, the observed resistance levels suggested that the 1bp overlap (i.e. '-1') gives the best expression level of the three configurations. Confirmation that the translation of the two ORFs is indeed coupled was obtained by analysis of the transgenic lines containing the cpl1 nonsense mutation. Namely, expression of aphA6 is abolished if cpl1 translation does not extend to the true stop codon, but terminates at the mutation. Recently, we have made more 'two-ORF' constructs to test the generality of the system. Firstly, the aphA6 and cpl1 ORFs were switched around (with a '-1' overlap) so that aphA6 was translated first. This gave rise to kanamycin resistant lines, but unfortunately the Cpl1 protein could not be detected by western blotting. Similarly, two transgenic lines were made in which ORFs encoding the E. coli lac repressor and human growth hormone (both have been successfully expressed in the chloroplast as separate genes) were combined with '-1' and '+1' overlaps. However, as before, only the gene product of the first ORF accumulated to detectable levels. Further work is now in progress in which we are testing short linker sequences between ORFs to see if this can improve expression of the second ORF.

D4-4: Chlamydomonas nuclear mutants affected in chloroplast proteases

i) The role of FtsH (partner 4). FtsH is the major transmembrane protease in the thylakoids, and an FtsH mutant was isolated by a suppressor approach. This mutant has been used extensively to study the action spectrum of FtsH. It was found that the primary roles of FtsH are (i) the recycling of photosynthesis proteins in stress conditions and (ii) the quality control of misassembled protein complexes. The substrate spectrum of the FtsH protease is currently under investigation. Besides PSII degradation in photoinhibitory conditions, FtsH proved to be involved in PSII degradation in nutrient starvation conditions, whether it being the absence of phosphate or of sulphur. FtsH is also involved in the degradation of cyt. b6f during nitrogen starvation, as well as most of the proteins specifically involved in its biogenesis. FtsH also controls the disposal of cyt. b6f complexes lacking heme ci and/or heme bh.

ii) Altered expression of the RNAse J, a chloroplast-based 5'-exo/endonuclease (partner 4). RNAi vectors for attenuating RNAseJ expression, a major 5'-3' chloroplast exonuclease have been devised and were used in nuclear gene transformation assays. Altered patterns of

maturation of polycistronic transcripts were observed. How these alterations reflect on the level of expression of the corresponding protein products remains to be investigated.

iii) Remodeling of the photosynthetic apparatus upon nitrogen starvation (partner 4). We showed that cytochrome b6f and most cytochrome b6f-related proteins involved in its biogenesis are selectively lost when Chlamydomonas is starved of a nitrogen source. This nutrient stress response was investigated further because it is the most commonly used procedure with Chlamydomonas for biotechnological approaches aimed at biofuel production. It was shown that a deep remodelling of the photosynthetic membrane develops with the time of nitrogen deprivation. The membrane loses its ability to contribute to CO2 fixation, not only because of the selective degradation of cyt b6f complexes but also because of the degradation of stromal RubisCO. In addition, prolonged illumination under nitrogen stress leads to the photo-destruction of the two photosystems. In parallel there is a three times increase in the rate of chlororespiration that is borne by a similar increase in the concentration of the major chlororespiratory enzymes, NDA2 and PTOX2. Thus the energy producing thylakoid membranes convert into an energy dissipating template upon nitrogen starvation, which emphasizes an unexpected metabolic flexibility of the photosynthetic membrane.

The process of activation of this degradation pathway upon nitrogen starvation was further investigated and a key role of NO in this signalling process was revealed. Genetic evidence was obtained to suggest that NO originates from an intracellular source of nitrite that develops at an early stage of nitrogen starvation.

D4-5 (a): Chlamydomonas strains with enhanced chloroplast transgene expression

In this project partners 8 and 9 have developed complementary genetic screens to isolate mutant strains that show either enhanced transgene expression or improve stability/accumulation of recombinant proteins in the chloroplast.

i) Chlamydomonas strains with enhanced chloroplast transgene expression (partner 8). The levels of transgene expression obtained in the chloroplast of Chlamydomonas have been highly variable depending on the protein of interest and on the regulatory elements used for its expression. Although the limiting factors are not well understood, it is clear that negative feedback regulation of gene expression at the post-transcriptional level plays a role, and at least in some cases, proteolytic degradation of the protein product (Specht et al., 2010; Michelet et al., 2011).

In order to devise a genetic screen for mutations that enhance transgene expression in the chloroplast, the chosen strategy was to create strains with two reporters under the control of the same chloroplast promoter/5' UTR, either psaA or psaB. With this dual system, it should be possible to distinguish mutations in trans-acting factors that will affect both reporters, rather than cis-acting mutations that will affect only one of the two constructs. For this purpose a new selectable marker was developed based on the bacterial aacC gene that confers moderate levels of gentamycin resistance (100 mg/L) to the Chlamydomonas transformants. Homoplasmic lines were first obtained that were transgenic for the psaB:aacC and psaA:aacC marker inserted at the atpB locus (atpB-int vector) (Michelet et al., 2011). The psaB:aacC line was then transformed with the second reporter, the firefly luciferase psaB:lucCP in the IR-int vector, however homoplasmic lines could not be recovered. As an alternative, it was next attempted to introduce the psaB:lucCP reporter at a different locus (tscA). This time homoplasmic transformants were obtained but they did not exhibit luciferase activity. In the case of psaA, an intron-less version of the psaA gene was first introduced, to avoid the recovery of mutants deficient in trans-splicing of this mRNA. Such mutants are relatively frequent and show loss of the negative feedback exerted by PsaA. This would lead to an indirect enhancement of reporter expression in our genetic screen (Wostrikoff et al., 2004; Michelet et al., 2011). The psaA:lucCP construct was then introduced, but again luciferase activity was not detected. Partner 8 is continuing to investigate whether intact lucCP constructs are present in these lines. When this is confirmed, the strains will be mutagenized and screened for enhanced gentamycin resistance and recovery of luciferase activity.

ii) Chlamydomonas strains with enhanced chloroplast transgene expression (partner 9). This alternate screen involved first creating a transformant line in which the accumulation of the kanamycin-resistance protein AphA6 is compromised by the addition of an N-terminal extension, leading to a lower resistance level compared to the native AphA6. This transformant was then used to make a double transformant in which a HA-tagged version of the aadA gene encoding spectinomycin resistance was placed at a neutral locus downstream of psaA-3. This double transformant is resistance to both kanamycin (100 µg/ml) and spectinomycin (1000 µg/ml), and was used in a UV-mutagenesis screen in which the aim is to select for increased kanamycin resistance and then score for increased AadA protein by western blot (the high level of spectinomycin-resistance conferred by the highly active AadA precludes scoring for any increased resistance). A large number of mutants showing high Km resistance have now been isolated. To date, western analysis of the first 20 have been failed to detect significantly increased levels of the AAD, but a screening of larger numbers using a high-throughput protein dot-blot method is in progress.

In addition to the mutant screen, partner 9 also investigated whether chloroplast expression of a chaperone from E. coli (termed Spy) would improve the accumulation of recombinant proteins. A codon-optimized and HA-tagged version of the Spy gene was synthesized and introduced into the chloroplast under the control of the psaA-3 promoter/5'UTR. Synthesis

of the chaperone was confirmed by western blotting with anti-HA antibodies. However, because of concerns that the HA epitope might interfere with substrate binding, a non-tagged version of the gene construct was also made and used to transform a Chlamydomonas line expressing the Verde fluorescent protein (VFP). These Spy/VFP transformants have been made and will be assessed for increased VFP protein and fluorescence levels in the next few weeks.

D4-5 (b): Transgene expression in protease-defective mutant strains

i) Expression in an FtsH protease deficient mutant (partners 4 and 9). To investigate whether the FtsH protease may degrade transgenic proteins and hence lower their level of expression, the ftsh1 mutant was crossed with lines expressing transgenic reporter proteins in the chloroplast. The following were used as reporters: the vapA gene encoding a surface antigen of Aeromonas salmonicida, the aacC gene that confers moderate levels of resistance to gentamycin, recently developed in this project, and the firefly luciferase gene lucCP (Matsuo et al., 2006). Proteolytic degradation was previously shown to limit VapA levels. The transgenes were placed under the control of the strong promoter / 5'UTR of the psaAex1 gene. To increase the expression of vapA, the mutant raa-L121G was used since it fails to trans-splice psaA mRNA and therefore does not accumulate PsaA. In this context the expression of the psaA:vapA construct is enhanced both at the mRNA and the protein level, probably because CES feedback inhibition by unassembled PsaA is relieved (Michelet et al., 2011).

Whether the stability of VapA is increased in the absence of this protease was examined using a genetic cross to ftsh1-1. However the amounts of VapA did not increase in the ftsh1-1 context, and on the contrary were unexpectedly lower that in the wild-type or raa-L121G. Similarly, a cross of the protease mutant with the psaA::aacC reporter line but did not result in any observed difference in the ability to grow on media containing different concentrations of gentamycin between the wild-type and the ftsh1-1 progeny. Finally, a similar cross of the ftsh1-1 protease mutant to the psaA::lucCP reporter was performed, and the progeny assayed for luciferase activity. No significant differences in luciferase expression between the wild-type and ftsh1-1 progeny were observed. These data indicate that expression of the three reporters is not significantly limited by FtsH-mediated degradation. This may be due to the localization of these recombinant proteins in the soluble fraction. Further genetic crossing or chloroplast transformation combining the ftsh1-1:{psaA:vapA} strain with mutation in a chloroplast protease such as ClpP that preferentially degrades soluble substrates might prove successful to over-accumulate proteins of interest.

D4-6: Chlamydomonas strains with efficient expression of biosynthetic genes for a C10 biofuel (partner 9).

As a proof-of-concept for the utilization of the chloroplast genetic engineering tools developed during the SUNBIOPATH project, it was planned to manipulate the terpenoid biosynthesis pathway with a view to producing the novel C10 terpenoid, geraniol.

This work is still in progress, but the following has been achieved so-far:

a) A cDNA clone encoding the plant enzyme geraniol synthase (GES) was obtained from Dr Eran Pichersky (Iijima et al., 2004), modified to remove the 5' coding region for the chloroplast targeting sequence and to add an HA-tag sequence at the 3' end, and then cloned into our chloroplast expression vector, pASapI.

b) The gene encoding the cyanobacterial enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS) was amplified from genomic DNA of Synechocystis PCC6803, modified to encode a C-terminal HA tag and cloned into a second chloroplast expression vector pSRSapI.

c) Homoplasmic chloroplast transformants have been generated for both constructs and western analysis has confirmed the presence of the DXS protein, but not the GES protein. An assay for DXS activity it currently being established.

WP5: Optimization and valorization of algal culture in photobioreactors

General objective. The optimized cultivation of algae in photobioreactors relies not only on efficient strains but also on precisely controlled physico-chemical parameters. Currently, there is a need for the critical evaluation of relevant parameters that control growth in photobioreactors. This has been achieved in this WP through the analysis of photosynthetic rates and macroscopic kinetic studies in lab-scale reactors. For the most interesting mutants that show a significantly improvement of growth rates and biomass yield (see WP2 and 3), growth in indoor reactors has been examined. Data obtained have been used to design a reactor built with cheap materials. Algal have been tested for biomethane production. Finally a techno economic and environmental analysis has been performed from the obtained results taking into account the C-credit of the whole process.

Objectives and achievements for the period

For the whole period, our objectives were to establish optimized parameters for algal growth in photobioreactors (goal 1, D5-1); to develop theoretical models describing the algal response to various light regimes (goal 2, D5-2); to design of a reactor that is based on cheap materials (goal 3, D5-3); to determine the efficiency of algal strains obtained by the

consortium for biogas production (goal 4, D5-4); to realize a techno economic and environmental analysis of algal strains obtained by the consortium (goal 5, D5-5) .

D5-1: Optimized parameters for algal growth in photobioreactors

The influence of variations in different parameters like temperature, pH, medium composition, CO2 supply (concentration/ partial pressure) and light intensity were investigated and optimized to determine the process conditions and possible ranges for control. For two wild types and different antenna-reduced mutants of Chlamydomonas reinhardtii (wt13, wt8b+, T7, as1, as2, bf4) growth kinetics was determined. The algae grow light limited up to intensities of about 300-500 µE/m2/s. Then photosynthesis becomes saturated at higher light intensities and could be even inhibited. 300-500 µE/m2/s light should be supplied to all algae cells within the whole reactor volume to operate production reactors with maximum efficiency. All mutants showed no lack in specific growth rate at low light densities (Jacobi et al., 2012; Lehr et al., 2012).

Further work was done to reach higher biomass densities of up to 6 g/L for optimum performance of antenna reduced mutants. This was evaluated in terms of photoconversion efficiency. The mutant bf4 showed the highest PCE value at middle and high light intensities. This is a clear proof of the value of the antenna reduction concept.

D5-2:Theoretical development of a dynamic model describing the response of algae to various light regimes

A photosynthesis-irradiance curve was measured under continuous illumination and used to calculate the net oxygen yield on light energy, which was maximal between a PFD of 100 and 200 µmol m-2 s 1. Net oxygen yield under flashing light was proven to be duty cycle dependent: the highest yield was observed at a duty cycle of 0.1 which corresponds to a time-averaged PFD of 115 µmol m-2 s-1. At lower duty cycles maintenance respiration reduced net oxygen yield. At higher duty cycles the specific photon absorption rate exceeded the maximal photon utilization rate and, as a result, surplus light energy was dissipated as heat. This behaviour was identical with the observation under continuous light.

These validation experiments showed that the underlying assumption of a reductant pool correctly describes photosynthesis under flashing light. The filling and emptying of the pool is depending on the flash time. With increase in flash time the energy dissipation rate increased as well. The model simulations showed that if the dark time between flashes is not

sufficiently long the electron pool will not be completely emptied and this results in an increased dissipation of absorbed light energy. The current model provides a good basis to simulate microalgae growth in real photobioreactors which are characterized by mixing-induced light/dark cycles in the range of 1 to 25 Hz (Vejrazka et al., 2011, 2012; Vejrazka et al., 2013).

D5-3: Design of a mass reactor that is based on cheap materials

To bring these advantages into action, a wave-surface-reactor has been developed. The reactor is a surface structured horizontal reactor. Horizontal reactors have different advantages but the basic disadvantage is that light dilution is only possible to a limited extend. The antenna-reduced mutants however, allow for this new reactor design.

D5-4: Efficiency of algal strains obtained by the consortium for biogas production

In order to maximize valorization of the whole biomass, the consortium also looked for the potential of several microalgae (Chlamydomonas, Scenedesmus, Euglena, Dunaliella,…) as alternate substrate for biogas production. The potential of these species to serve as a substrate for biomethane production differs greatly and is for instance dependent on the rigidity of the cell wall as a determinant of digestibility. Fermentation of the green alga Chlamydomonas was most efficient. We conclude from our results that selected algae species represent good substrates for biogas production. In addition, to evaluate integrative biorefinery concepts, hydrogen production in Chlamydomonas prior to anaerobic fermentation of the algae biomass was measured and resulted in a 20% increase of biogas generation. We conclude that selected algae species can be good substrates for biogas production and that anaerobic fermentation can seriously be considered as final step in future microalgae-based biorefinery concepts.

D5-5: Techno economic and environmental analysis of algal strains obtained by the consortium

A reduction of specific absorption by the biomass leads to a reduction of non-photochemical quenching at the front side of the reactor, what is the first and main reason for light saving. Along the light path the light intensity is higher compared to the wild type case also leading to better growth and a reduced fraction of respiration. Experiments as given in deliverable 5-1 showed that these effects have been accumulated to a doubling of PCE for the antenna reduced mutant bf4 under high light conditions and high biomass concentration. This is a

clear prove that the concept of antenna reduction leads to a direct effect in saving energy for biomass production. A reduction of the CO2 footprint for algal biomass production is possible by about 30%.

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Potential Impact:

1. Strategic impacts

Potential impact for culturing algae

Culturing algal cells in photobioreactors requires high-density cells at high light intensities in order to get the maximum photosynthesis efficiency and biomass productivity. The consortium demonstrated that small antenna size mutants represent good options to maximize growth in these conditions since photoconversion efficiency and biomass productivity was doubled in high light and high density in the mutants compared to the corresponding wild type. The SUNBIOPATH consortium thus contributed very efficiently to the improvement of algal growth in photobioreactors, and allowed an evaluation as to whether metabolic engineering of Chlamydomonas can result in superior strains for solar-to-fuel energy conversion. This knowledge could be applied to Dunaliella, another microalgal species, used in the consortium and interesting for the production of glycerol and beta-carotenes.

This allowed the consortium to build a new wave-surface-reactor, which maximizes the growth of the antenna-reduced mutants. In addition, the consortium defined the most appropriate media to be used for growth in large-scale photobioreactors and built a dynamic model describing the response of the microalgae to various light regimes. This model provides a good basis to simulate growth in real photobioreactors, which are characterized by mixing induced light/dark cycles in the range of 1 to 25 Hz. Thus the SUNBIOPATH consortium also contributed to new developments at the level of bioengineering.

Techno economic analysis revealed a reduction of the CO2 footprint for algal biomass production by about 30% using these mutants.

In addition, the consortium demonstrated that the chloroplast has applications beyond biofuels and is a promising target for production of high value products by engineering multiple transgene expression.

SUNBIOPATH also demonstrated that microalgae (Chlamydomonas, Scenedesmus, Euglena, Dunaliella,…) are attractive as alternate substrate for biogas production. The potential of these species to serve as a substrate for biomethane production differs greatly and is for

instance dependent on the rigidity of the cell wall as a determinant of digestibility. Fermentation of the green alga Chlamydomonas was most efficient. To evaluate integrative biorefinery concepts, hydrogen production in Chlamydomonas prior to anaerobic fermentation of the algae biomass was measured and resulted in a 20% increase of biogas generation. We conclude that selected algae species can be good substrates for biogas production and that anaerobic fermentation can seriously be considered as final step in future microalgae-based biorefinery concepts.

In addition, the consortium contributed to the general knowledge about the growth conditions and transformation techniques of Dunaliella, a marine species and contributed by this way to the development of the maritime economy in Europe.

2. Wider societal implications

By its large audience dissemination activities including journal articles and interviews on public radio chains (see below), the SUNBIOPATH project contributed to a better knowledge of the 'algal world' in the public and by this way, the concept of using algae for energy, food and feed, reached a large audience, from very young people to adults.

3. Main dissemination activities

Dissemination to scientific audience

Peer-reviewed papers

Members of the SUNBIOPATH consortium published an impressive number of scientific articles in international peer-reviewed journals, such as Plos Biology, Plant Cell, Proc Natl Acad Sci (USA), Plant J, Plos One, and applied and industrial journals such as Journal of Biotechnology, Engineering of Life Sciences, Biotechnology Bioengineering. Altogether, these articles contributed very efficiently to the dissemination of the knowledge of SUNBIOPATH to the scientific community.

Book chapter

In addition, the members of the SUNBIOPATH consortium participated to the writing of a book chapter (Bassi et al. 2011, Finding the bottle-neck: A research strategy for improved

biomass production, in Microalgal Biotechnology: Integration and Economy, C. Posten and C. Walter, Editors. 2012, De Gruyter: Berlin. p. 227-52), in which the reader will find the main achievements of SUNBIOPATH. Written in a book co-edited by one of the members of SUNBIOPATH (Clemens Posten) and covering the present day microalgal biotechnology knowledge, this chapter represents a very efficient way to reach scientific from different fields, as well as economists and stakeholders.

Participation to scientific meetings

In addition, members of the SUNBIOPATH consortium including PI and potsdocs hired by the project participated to numerous international scientific conferences all over the world and organized meetings and workshops, and this contributed very efficiently to the dissemination of the project.

Participation to industrial meetings

In addition, the SUNBIOPATH project was presented at industrial events such as the European Biomass Conference held in Berlin in June 2011 or the Algae Europe held in Milano in October 2011.

Organization of an international meeting at the end of the project

One of the achievements of SUNBIOPATH is the organization of a joint international meeting with two other KBBE-funded projects, BAMMBO and GIAVAP. The meeting was held in Brussels on January 21-23 2013.

GIAVAP (Genetic Improvement of Algae for Value Added Products) and BAMMBO (Sustainable production of Biologically Active Molecules of Marine Origin) are two European projects funded in 2010 by KBBE. Together with Stefan Leu from GIAVAP and Daniel Walsh from BAMMBO, a joint meeting was organized that gathered around 60 people from the three consortia as well as Tomasz Calikowski, project officer of GIAVAP and BAMMBO. As described below, the meeting was organized in twelve sessions that combined the results of the three projects. In total, 42 presentations were shown. In addition, external speakers and members of the advisory boards of the projects (Prof R Bock from Max Planck Institute of Golm, Prof Arthur Grossman from the Carnegie Institution for Science - Standford, Dr Thomas Kyi Global Head Strategic Business Development - Lonza) also gave plenary lectures

and provided valuable feedbacks about the meeting and the future of the algal research. This gave the unique opportunity for discussions among the different groups and was highly profitable to all of them. The meeting went through all the aspects of the algal research and was a demonstration that both basic and applied sciences as well as technologies are needed to shape the algal research and their applications.

Dissemination to public audience

The SUNBIOPATH project was presented in a Belgian weekly newspaper (Le Vif l'Express) and in the FNRS-News edited by the Fonds de la Recherche Scientifique from Belgium. The project was also presented at the occasion of an interview of C Remacle by Véronique Thyberghien, about the utilization of microalgae to produce electricity [27/03/13, Program 'Ô positif' of the RTBF (Radio Television Belge Francophone)].

Oral presentations

Several oral presentations to public audience have contributed to the dissemination of the SUNBIOPATH project. The target audience was stakeholders of regional governments or general audience.

The project also made synergies with Science Education by presenting the SUNBIOPATH project at the occasion of the 'Plant Fascination Day' organized by EPSO in the universities of Geneva and Liege on May 18 2012 as well as during the 'Researcher's Night' in September 2011 in Liege.

4. Exploitation of results

To use the specific advantages of antenna-reduced mutants, specific reactor designs are possible. Light dilution for the developed mutants can be reduced by a factor 2, which allows for the reduction of the surface to footprint area by this factor. Therefore, the material costs can be cut by half. In addition, cheaper materials are possible in thinner layers by reduced hydrodynamic pressure.

To bring these advantages into action, a wave-surface-reactor has been developed under the framework of the project. The reactor is a surface structured horizontal reactor. Horizontal reactors have different advantages but the basic disadvantage is that light dilution is only possible to a limited extend. The antenna-reduced mutants however, allow for this new reactor design. This led to a new patent by partner 10 (EP 2 388 310 A1) who also participates to another European project "KIC inno energy" where a demonstration specimen will be built.

Another advantage of the use of these mutants is that the techno economic analysis revealed a reduction of the CO2 footprint for algal biomass production by about 30%.

List of Websites:

http://www2.ulg.ac.be/genemic/SUNBIOPATH/