Microalgal lipid biosynthesis - Phylogeny of acetyl-CoA carboxylase and gene expression patterns of key enzymes

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Abstract

Lipids from microalgae have become a valuable commodity in the last 20 years. Two of the potential industrial applications are the production of biodiesel from short-chain saturated and monounsaturated fatty acids (FAs) and the use of very long-chain polyunsaturated fatty acids (VLC-PUFAs) to supplement human / animal diets. However, high production costs are still prohibitive and an increase in yield is desired. Understanding the cellular mechanisms by which microalgae produce these FAs and how these mechanisms can be manipulated to potentially increase yield is of immediate interest to the industry. Generally, the lipid content of algae increases under nitrogen starvation, whereas FA length and desaturation decreases. While the general biochemical processes are known, research investigating the expression of the different enzymes involved under different growth conditions and growth phases is still lacking. This kind of knowledge will be invaluable for targeted manipulations of environmental conditions with the aim to increase yields of desired types of FAs for different applications.

In algae, *de novo* synthesis of FAs up to C18 occurs in the plastids, where the key enzyme acetyl-CoA carboxylase (ACCase) catalyses the first committing step. Further elongation and desaturation of FAs to VLC-PUFAs is associated with the endoplasmic reticulum (ER). At this step, cytosolic ACCase provides malonyl-CoA for the elongation of fatty acids in the ER. In addition to the two locations, there are two types of ACCase: the bacterial, multi-subunit heteromeric ACCase and the eukaryotic multi-domain homomeric ACCase. While cytosolic ACCase in eukaryotes is always homomeric, plastidial ACCase, if present, can be either heteromeric or homomeric. The multi-domain nature of homomeric ACCase makes it easier to study and manipulate and shows great potential for improving FA synthesis and elongation. It is therefore important to identify the type of ACCase present in the plastids of algae, to enable gene expression studies to be conducted. The two main aims of this thesis were: 1) to increase the general knowledge of ACCase in algae and provide a phylogenetic analysis of the relationships between algae in terms of ACCase; 2) to correlate the gene expression of plastidial and cytosolic ACCase and selected desaturases under nitrogen-deplete and nitrogen-replete growth conditions during different growth phases with FA content and composition data.

Despite the recognition of the existence of heteromeric and homomeric ACCase forms, generalised published statements still list the heteromeric form as the one present in

algal plastids. In the present research, heteromeric and homomeric ACCase amino acid sequences obtained from the National Center for Biotechnology Information (NCBI) and the Joint Genomic Institute (JGI) were synthesized with taxonomic data. This elucidated that the presence of heteromeric or homomeric ACCase is dependent on the origin of the plastid. Plastids derived from a primary endosymbiotic event (Chlorophyta, with the exception of the green algal class Prasinophyceae, and Rhodophyta) contain heteromeric ACCase. In contrast, plastids derived through secondary or tertiary endosymbiosis (Chlorarachniophyta, Cryptophyta, Stramenopiles, Haptophyta, and apicoplast (rudimentary plastid) containing Apicomplexa), contain homomeric ACCase. This highlights that the majority of algae actually contain homomeric plastidial ACCase.

To reconstruct a Bayesian inference tree, five novel sequences of homomeric ACCase from *Isochrysis* aff. *galbana* (TISO), *Nannochloropsis oculata* and *Chromera velia* were sequenced and used together with all other publicly available amino acid sequences of algal ACCases to date. The cytosolic and plastidial ACCase sequences of the Stramenopiles, Alveolates and Rhizaria (SAR) formed two strongly supported clades. These clades agree with the recently proposed new phylogeny from the literature, where the Rhizaria are related to the Stramenopiles and Alveolates. In addition, the plastidial ACCase clade also included the Prasinophyte sequences, while the cytosolic ACCase clade containing the SAR species excluded the Prasinophytes. This shows that, while the ancestor hosts of the SAR and Prasinophytes are not closely related, they acquired the plastidial homomeric ACCase from the same source and not through a duplication event from either host.

To investigate the expression of selected genes at different growth phases, *I.* aff. *galbana* (TISO) and *Chromera velia* were cultivated over 12 days in nitrogen-replete and nitrogen-deplete conditions. Cell density and nutrient status were measured daily, while gene expression, dry weight and lipid content were measured during the logarithmic, late logarithmic and stationary growth phases. The cell density of *C. velia* of cultures grown with and without nitrogen did not differ notably during the first four days of culture. In contrast, nitrogen-replete cultures of *I.* aff. *galbana* (TISO) exhibited higher cell densities from the first day of culturing, compared to cultures grown in nitrogen-deplete conditions. During active growth, FA content is generally considered to be low, since the FA content is shared by the daughter cells at cell division. In terms of gene expression patterns of plastidial ACCase and lipid content, distinct differences were observed between *I.* aff. *galbana* (TISO) and *C. velia*. The expression of plastidial ACCase of *C. velia* was significantly upregulated during the late logarithmic and

stationary phases in both types of cultures, and nitrogen-deplete cultures showed a more pronounced response to nutrient starvation (N+: 2-fold; N-: 4-fold). The upregulation of plastidial ACCase expression strongly correlated with an increase in FA content. Conversely, *I.* aff. *galbana* (TISO) showed a significant 8-fold upregulation of plastidial ACCase during the logarithmic growth phase of nutrient-replete cultures only, consistent with higher growth rates. Since the FA content of *I.* aff. *galbana* (TISO) did not vary significantly between treatments and growth phases, the upregulation of plastidial ACCase can be explained by the algae maintaining a stable FA content during active growth. Finally, the expression of cytosolic ACCase in *C. velia* decreased by approximately half with culture age, independent of nutrient status. This decrease correlated well with an average increase in short-chain fatty acids (≤C18) of approximately 240%, showing that cytosolic ACCase plays an important part in supplying the elongases located in the ER with malonyl-CoA for the elongation of FAs beyond C18.

Isochrysis aff. *galbana* (TISO) has been used extensively in aquaculture because of its high content of docosahexaenoic acid (DHA, C22:6n-3), a nutritional supplement important for the health of most aquaculture animals. Therefore, the expression of several desaturases (d6FAD, d8FAD, d5FAD and d4FAD) involved in the desaturation and elongation pathway were measured. Similar to the total FA content, the average DHA content of 12.2 mg g^{-1} ash-free dry weight did not vary significantly between treatments and growth phases. The expression of d6FAD was not significantly upregulated at any time. In contrast, d8FAD, which provides a bypass of the normal d6FAD pathway, was upregulated 7-fold during logarithmic growth of nutrient-replete cultures. Correspondingly, the expression of d5FAD and d4FAD were upregulated 8.5-fold during logarithmic growth. None of the intermediate FAs in the DHA synthesis pathway were present at high concentrations, suggesting an efficient conversion at each step of the pathway. Even though there was no increase in total DHA content analogous to the increase in gene expression, it is important to keep in mind that the cells were actively growing during this phase. The upregulation of these desaturases therefore allowed the microalgae to maintain adequate levels of the membrane FA DHA during active growth.

In summary, the present thesis provides the first comprehensive review of the plastidial ACCase type in microalgal phyla and classes. This is of great importance in terms of algal phylogeny, but also for further investigations of algal lipid synthesis. Moreover, it was shown that growing algae in nitrogen-deplete conditions does not always result in an accumulation of FAs, although there is a strong correlation between the expression

of plastidial ACCase and FA production. Similarly, even thought the DHA content in *I.* aff. *galbana* (TISO) did not differ between growth phases, the involved desaturases were highly upregulated during logarithmic growth to maintain membrane FA DHA levels. Additional research should focus on microalgal strains with differences in their VLC-PUFA content between growth phases, to further investigate the differences in gene expression between different algal species.

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Abbreviations

α-CT	α-carboxyltransferase
ß-CT	ß-carboxyltransferase
aa	Amino acid
AAT	Acyl-ACP thioesterase
ACC1	Plastidial ACCase in algae
ACC2	Cytosolic ACCase in algae
ACCase	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ACS	Acyl-CoA synthase
AFDW	Ash-free dry weight
ALA	α-linoleic acid
ARA	Arachidonic acid
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
BI	Bayesian inference
CER	Chloroplast endoplasmic reticulum
CMGP	Cyanidioschyzon merolae Genome Project
CoA	Coenzyme A
CT	Carboxyltransferase
d4FAD	Δ4-desaturase
d5FAD	Δ5-desaturase
d6FAD	Δ6-desaturase
d8FAD	Δ8-desaturase
DAG	Diacylglycerol
DGAT	Acyl-CoA:diacylglycerol acyl-transferase
DGD	Digalactosyldiacylglycerol synthase
DGDG	Digalactosyldiacylglycerol
DGLA	Di-homo-γ-linoleic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DSN	Duplex-specific thermostable nuclease
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ETA	Eicosatetraenoic acid
FA	Fatty acid
FAD	Fatty acid desaturase

FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLA	γ-linoleic acid
GPAT	Acyl-CoA:glycerol-3-phosphate acyl-transferase
HGT	Horizontal gene transfer
HTL	Hydrothermal liquefaction
JGI	Joint Genome Institute
L	Logarithmic growth phase
LA	Linoleic acid
LL	Late logarithmic growth phase
LPA	Lysophosphatidate
LPAAT	Lysophosphatidate acyl-transferase
MGD	Monogalactosyldiacylglycerol synthase
MGDG	Monogalactosyldiacylglycerol
ML	Maximum likelihood
N-	Cultures grown under nitrogen-deplete starting conditions
N+	Cultures grown under nitrogen-replete starting conditions
NCBI	National Center for Biotechnology Information
nt	Nucleotide
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
plPDC	Plastid pyruvate dehydrogenase complex
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
S	Stationary growth phase
SACPD	Stearoyl-ACP desaturase
SAR	Stramenopile, Alveolata, Rhizaria
SC-FA	Short-chain fatty acid
SDA	Stearidonic acid
SE	Standard error
TAG	Triacylglyceride
TE	Transesterification
VLC-FA	Very long-chain fatty acid
VLC-PUFA	Very long-chain polyunsaturated fatty acid

Chapter 1 - General introduction

1. Fatty acids as a renewable source

Modern society is heavily dependent on fuel for transportation, essential nutrition for our general wellbeing, and bio-based polymers. The increasing world population, and therefore increasing demand for these products, makes the development of new sustainable resources a priority. In addition, carbon neutral or negative production systems are desirable, if climate change is to be taken into consideration. Phototrophic organisms like plants, algae and cyanobacteria have an immense potential as renewable resources to alleviate many of the current issues.

In terms of versatility, fatty acids are the most interesting photosynthetic product, having applications in human nutrition, and the production of renewable fuels and plastics. Oxygenic photolithotrophic organisms use solar energy, CO_2 and H_2O to produce organic molecules (e.g. amino acids, nucleotides, carbohydrates and lipids) and O_2 through oxygenic photosynthesis. Of these organic molecules, lipids are the most highly reduced, and consequently have the highest energy density (Durrett et al., 2008). Lipids can take a variety of forms; the most common lipids are polar membrane lipids and neutral triacylglycerides (TAGs), which are made up by one glycosylglyceride, phosphoglyceride, or glyceride molecule esterified with two to three molecules of fatty acids (FAs). FAs can measure between 4 and 28 carbon atoms in length and are made up of a carboxylic group with a long, generally unbranched, aliphatic tail. The aliphatic tail can contain a variable number of double bonds, in which case the FAs are called unsaturated, while saturated FAs do not contain double bonds in their carbon chain. Most FAs of plants and algae tend to have an even number of carbon atoms and the double bonds of unsaturated FAs mostly occur in cis-configuration, resulting in a curved tail. The length and number of double bonds affect the chemical and physical properties of the FAs. FAs are very versatile in their application, depending on their length and level of unsaturation. Long-chained polyunsaturated fatty acids are an essential component of human nutrition (Adarme-Vega et al., 2014), short-chained, saturated or mono-unsaturated FAs can be used to produce fuel for transportation in the form of biodiesel (Atabani et al., 2012), while a variety of FAs are used in the production of renewable polymeric materials (Lligadas et al., 2013).

The FA nomenclature used in this thesis will take the form of CX:Yn-Z, where X is the total number of carbon atoms, Y the number of double bonds and Z the position of the first double bond counted from the terminal methyl carbon towards the carbonyl carbon. Due to the importance of the position of the last double bond in the aliphatic

chain, FAs will be categorized into ω-3 and ω-6, where appropriate, which again refers to the first double bond from the terminal methyl carbon end.

2. Fatty acids in nutrition

As an essential nutritional component for humans, very long-chain polyunsaturated FAs (VLC-PUFAs) positively affect cardiovascular health (Delgado-Lista et al., 2012, Vlachopoulos et al., 2013), inflammation (Kiecolt-Glaser et al., 2012, Rangel-Huerta et al., 2012), infant development (Luchtman and Song, 2013, Gil-Campos and Sanjurjo Crespo, 2012, Swanson et al., 2012), learning and behaviour (Kuratko et al., 2013), and neurodegenerative diseases (Crupi et al., 2013, Swanson et al., 2012). VLC-PUFAs for human consumption are currently mainly obtained from oily fish species like herring, mackerel, sardine and salmon (Gunstone, 1996), with the demand for VLC-PUFAs soon expected to exceed current yields from aquaculture and fisheries (Sijtsma and de Swaaf, 2004), especially considering the current overfishing of the oceans (Myers and Worm, 2003). FAs also form an important feed ingredient component for animals grown in aquaculture, with the amount and composition of fatty acids being an important nutritional quality factor (Fidalgo et al., 1998). For example, VLC-PUFAs are essential for the growth, development and survival of marine fish larvae (Eamta et al., 2003), shrimp (Cavalli et al., 1999) and molluscs (Langdom and Waldock, 1981, Knaur and Southgate, 1999). VLC-PUFAs therefore play an important role in human health, be it directly as a food supplement, or indirectly through the consumption of farmed species which were grown on a high VLC-PUFA diet. Unfortunately, oilseed plants mainly produce monounsaturated FAs (i.e. with one double bond), and the production of VLC- PUFAs in plants would therefore only be possible by using genetically modified organisms (López Alonso and García Maroto, 2000).

3. Fatty acids as fuel

Sustainable fuel supply for combustion engines is especially important for the transport industry, such as marine vessels (Lin, 2013) and aviation (Winchester et al., 2013), where other sustainable alternatives, like solar electricity, are often not suitable. There are a variety of processes to produce biofuels from algal biomass, each with different requirements. The traditional approach involves the direct production of biodiesel from dried algal biomass through transesterification (TE) of TAGs to FA alkyl esters, requiring large amounts of neutral lipids and not utilising the remaining biomass directly (Sheehan et al., 1998, Islam et al., 2013). Conversely, many modern processes are more efficient and make use of the entirety of the biomass to produce a bio-crude, which then can be fed into a traditional refinery to produce conventional fuels through

hydrocracking (Biller and Ross, 2011). For example, hydrothermal liquefaction (HTL) involves the treatment of wet biomass with high temperatures (300- 350 °C) and pressure (approximately 200 bar) (Biller and Ross, 2011). A recent study in *Nannochloropsis* sp. produced a bio-crude which contained 80 % of the original energy of the algal biomass (Valdez et al., 2012). However, the high temperature and pressure involved in HTL is energy intensive and requires more advanced equipment than TE, making TE more suitable for localised production of biofuels at remote biomass production facilities (Islam et al., 2013, Biller and Ross, 2011).

The lipid content and FA composition of the algal feedstock are important factors in biofuel production. For example, HTL of algal biomass with a high lipid content leads to better yields of bio-crude, compared to algal biomass high in proteins or carbohydrates (Biller and Ross, 2011). Furthermore, the fuel quality of biodiesel after TE depends on the characteristics of the various individual fatty acid alkyl esters, which are determined by their structural features (chain length, number of double bonds and chain branching) and the alkyl ester group (Knothe, 2005, Islam et al., 2013). Fuels with higher cetane numbers have shorter ignition delays, run more smoothly and have more complete combustion (Nelson, 1958). Biodiesel with high amounts of long-chained, or saturated FAs, exhibit a higher cetane number, but also have a higher cloud point (temperature where dissolved solubles start precipitating, which can clog fuel filters and injectors) and poor cold flow qualities (Pinto et al., 2005). Cold flow qualities are important for biodiesel used in cold climates (Nelson, 1958). In contrast, biodiesel high in unsaturated FAs has a low cetane number and poor oxidation stability (resistance to degradation through oxidisation), but does not have the disadvantages of saturated FA-based biodiesel mentioned above (Pinto et al., 2005). It is therefore important to produce biodiesel with correct mixtures of different FAs for maximal efficiency and a good exhaust profile. Oleic acid (C18:1 n-9) particularly exhibits a combination of desirable characteristics (Knothe, 2005, Islam et al., 2013). One suggestion for an optimal fuel mix is C16:1, C18:1 and C14:0 in a ratio of 5:4:1, resulting in good oxidative stability, while retaining good cold flow characteristics and a high cetane number (Schenk et al., 2008). These parameters, in addition to the presence of contaminants like glycerine, catalyst, alcohol and free fatty acids, are important for biodiesel quality standards, which differ among regulatory bodies (Knothe, 2006).

4. Fatty acid derived polymers and other products

Fossil feedstocks are not only used for fuel production, but also in the manufacturing of plastics, paints, adhesives and many other materials (Lligadas et al., 2013, Williams and Hillmyer, 2008). Fossil feedstock derived polymers are unsustainable in the long run, as they are affected by peak oil in the same way as fossil derived fuels, making the search for renewable feedstocks for polymer production essential. The use of bio-based FAs as alternative feedstocks in the chemical industry has many advantages, including high biodegradability, low cost, and low toxicity (Lligadas et al., 2013). These polymers derived from FAs have the potential to be as cost-effective as petroleum-based materials, with the added benefit of lower ecological impact (Williams and Hillmyer, 2008). Potential FA derived end products include lubricants, paints, cosmetics, pharmaceuticals, biomedical products, plasticizers and construction materials (Gallezot, 2012). The presence of a carboxyl group and double bonds provides a variety of possibilities for cross-linking. For example, double bonds can be epoxidised, which allows the reaction with other molecules for polymerisation (Lligadas et al., 2013, Gallezot, 2012). Currently, bio-based polymers are mainly derived from terrestrial plants (Lligadas et al., 2013).

5. Market sizes

Algae have a vast potential to replace unsustainable resources in the production of omega-3 FAs, fuel and polymers. The global market in omega-3 FAs was estimated at USD 1.6 billion in 2010, expected to reach USD 2.5 billion in 2014, and projected to reach USD 4 billion by 2018 (Report: *Global Omega 3 (EPA/DHA) Ingredients Market - Industry Analysis, Market Size, Share, Growth and Forecast, 2010 - 2018*; http://www.transparencymarketresearch.com). While North America constitutes the largest market for omega-3 FAs, the Asia-Pacific (East Asia, Southeast Asia and Oceania) market is considered to be the most promising in terms of growth. Of the expected omega-3 FAs production in 2014, 18.9% are anticipated to be produced by algae, while the algal contribution in the Asia-Pacific industry is expected to be 37.5 % (Report: *Global Analysis of the Marine and Algae Omega-3 Ingredients Market*; Frost & Sullivan; http://www.frost.com/sublib/display-report.do?id=N97E-01-00-00-00).

The demand for renewable fuels is rapidly increasing, with growing pressure to improve energy security and the implementation of renewable fuel standards that mandate the increasing percentage of renewable fuels blended into transportation fuel. The global biofuel market has been valued at USD 83 billion in 2011 and is expected to double by 2021 (Report: *Global biofuels market could double to $185.3 billion by 2021*; http://clea

ntechnica.com/2012/02/20/report-global-biofuels-market-could-double-to-185-3-billion-by-2021/#pmWB4exBJlGyOjwz.99). The biofuel market is currently dominated by ethanol produced from corn and sugar cane, which is not projected to change by 2021 (Report: *Biofuels markets and technologies*; http://www.navigantresearch.com/research/biofuels-markets-and-technologies).

The use of bio-based polymers is still comparatively small and is expected to account for approximately 1 % of the world-wide polymer production in 2015 (Babu et al., 2013). Nevertheless, the natural FA market for biopolymer production is showing a similar increase to omega-3 FAs, being valued at USD 7.2 billion in 2011, USD 6.9 billion in 2012, and is expected to reach USD 13 billion in 2017 (Report: *Global markets for oleochemical fatty acids*; www.bccresearch.com). Considering the total polymer industry is estimated to reach USD 567 billion in 2017 (Report: *Global Polymer Industry 2012 to 2017: Trend, Profit, and Forecast Analysis*; http:// http://www.lucintel.com), there is still a lot of potential in this market to expand on bio-based polymers.

The use of terrestrial plants as the main renewable feedstock for most biofuel and bio-based polymers, is unfortunately not completely sustainable, and could therefore become limited in future. Most terrestrial plants are dependent on arable land and directly compete with food production (e.g. Chen and Khanna (2013) and Zilberman et al. (2013)). For example, the production of ethanol from crops artificially inflates the prices of corn, wheat and rice (Bahel et al., 2013, de Gorter et al., 2013). Furthermore, the clearing of land, for example for the planting of oil palms for biodiesel production, increases the net output of greenhouse gases (e.g. Hallgren et al. (2013) and Timilsina and Mevel (2013)), and has a negative impact on biodiversity (e.g. Immerzeel et al. (2013) and Fitzherbert et al. (2008)).

6. Sources of fatty acids

As outlined before, there is a clear need for VLC-PUFAs for human nutrition, and FAs in general to replace dwindling fossil feedstocks for fuel and polymer production. However, the adequate supply of these FA based renewable resources in large enough quantities is problematic. It is therefore important to find renewable sources that do not directly compete with food production and do not require the clearing of vast land areas. Microalgal production systems are independent of arable land and brackish water can be used for many species, removing the direct competition with food production. In addition, waste CO_2 from power plants, cement kilns, landfills and anaerobic digesters can be used directly to enhance production (Sheehan et al., 1998). Many microalgae are naturally high in FAs, especially VLC-PUFAs (Martínez-

Fernández et al., 2006, Huerlimann et al., 2010). Furthermore, microalgae are highly efficient in converting solar energy into algal biomass (up to 10%), and have a high lipid content, commonly 20-50% of dry weight (Sheehan et al., 1998, Spolaore et al., 2006, Rittmann, 2008, Schenk et al., 2008). There are additional benefits of microalgae compared to plants. Microalgae tend to have short doubling times (0.35 - 1 day) and harvesting can be continuous (independent of seasons) (Rittmann, 2008). This results in up to 20 times higher lipid production per unit area of land compared to terrestrial oilseed crops (Chisti, 2008, Rittmann, 2008, Sheehan et al., 1998).

7. Algal lipid synthesis pathways

Until recently, details on the lipid synthesis pathways in algae were limited. Most information available was scattered within the literature. Chapter 2 provides a detailed description of the current state of knowledge of the lipid synthesis pathways in algae. Accordingly only a short summary will be provided here.

Due to their close evolutionary relationship of their plastids, it is assumed that the plastidial biochemical pathways in plants and algae are similar (Livne and Sukenik, 1990, Roessler, 1990, Roessler et al., 1994). In plants, the *de novo* synthesis of FAs and the synthesis of galactolipids are located in the plastids, while the desaturation and elongation of FAs, and the synthesis of TAGs and phospholipids are located in the smooth endoplasmic reticulum (ER). FAs up to C16 and C18 are synthesised in the plastid by fatty acid synthase from malonyl-CoA, which is in turn synthesised by acetyl-CoA carboxylase (ACCase) from acetyl-CoA. Due to its location at the beginning of the *de novo* FA synthesis pathway, ACCase is considered a key enzyme in the production of FAs (Ohlrogge and Jaworski, 1997) and therefore provides an interesting target to investigate lipid synthesis in algae. In plants, ACCase occurs in two different locations: 1) in the cytosol, where it is involved in the elongation of FAs, and 2) in plastids, where it is involved in *de novo* FA synthesis (Nikolau et al., 2003). In addition to the two locations, there are two forms of ACCase: 1) heteromeric ACCase of prokaryotic origin, and 2) homomeric ACCase of eukaryotic origin. While the cytosolic ACCase of eukaryotes is always homomeric, algal plastids can potentially contain the heteromeric or homomeric form of ACCase, depending on the evolutionary history of the algae in question. While ACCase provides an interesting target for *de novo* FA synthesis in the plastid and FA elongation in the smooth ER, the enzyme is not involved in the desaturation of FAs. If an increase in VLC-PUFAs is desired, research should also investigate desaturases, which introduce double bonds at specific locations in FAs. In

general, questions about lipid synthesis, elongation and desaturation can be loosely divided into fundamental science and industrial applications.

8. Fundamental science

Algae show great taxonomic diversity, with new species still being described (De Clerck et al., 2013). The presence of several endosymbiotic events, where a heterotrophic cell took up a cyanobacterium (primary) or phototrophic eukaryotic organism (secondary or tertiary), complicates the phylogenetic investigation of algae. In addition to endosymbiotic gene transfer, gene duplication and horizontal gene transfer events further confound phylogenetic analyses. As a result, algal phylogeny is still not completely resolved (Bodył et al., 2009, Keeling, 2010).

Eukaryotic life is currently divided into six major supergroups, Opisthokonta, Amoebozoa, Archaeplastida, Rhizaria, Chromalveolata and Excavata (Adl et al., 2005, Keeling et al., 2005), of which the last four contain photosynthetic members. The Archaeplastida include the Viridiplantae (land plants and green algae), the Rhodophyta (red algae) and the Glaucocystophyta (a small group of freshwater microalgae) (Ball et al., 2011). There is strong evidence that the plastids of these three groups evolved from a single primary endosymbiotic event involving a cyanobacterium (Archibald, 2012). The majority of algal diversity, however, appears to be a testament to secondary and tertiary endosymbiotic events. At least three secondary endosymbiotic events gave rise to a large number of highly diverse organisms with plastids derived either from a red or green alga. The red lineage, first proposed by Cavalier-Smith (1998; 1999), is composed of the Chromalveolata, joining the former supergroups Chromista (containing the Cryptophyta, Haptophyta and Stramenopiles) and Alveolata (comprising of Apicomplexa, Chromerida, Ciliophora and Dinoflagellata). The green lineage is comprised of the Chlorarachniophyta (Rhizaria) and Euglophyta (Excavata). Even though the phylogenetic relationships within these phyla are better resolved, the relationships on a broader evolutionary level are still contested.

As mentioned above, algae potentially contain heteromeric or homomeric ACCase in their plastid. However, the presence of either form in the plastids of algae has not been well documented and needs revision. Publications mentioning ACCase in algae often cite heteromeric ACCase to be present in the plastid (e.g. Guarnieri et al. (2013)). Unfortunately, a thorough investigation of the presence of heteromeric and homomeric ACCase in the plastids of algae has not been performed to date. Furthermore, the dual locations of ACCase (cytosolic and plastidial) and the possibility of various origins of the plastidial form may provide a way to further unravel the unresolved phylogeny of

algae. Lastly, even though studying the phylogeny of algae is mainly fundamental science, there are also industrial applications, as outlined below.

9. Industrial applications

While the production of biodiesel from algae is technically feasible, it is not yet economically viable and an increase in lipid productivity is of importance for the industry (Griffiths and Harrison, 2009). Unfortunately, the high lipid content of algae does not necessarily equate to high lipid productivity. There is a strong negative linear relationship between lipid content and biomass productivity (Fig. 1.1). Algae with high lipid content tend to have slow growth rates and *vice versa*. The conditions under which algae accumulate lipids can provide an explanation to this observation. During logarithmic growth, most lipids are glycerol-based membrane lipids (polar lipids such as glycosylglycerides and phosphoglycerides) (Hu *et al.* 2008). In contrast, TAGs are used by microalgae to store energy and have no structural function (Harwood & Jones 1989, Hu *et al.* 2008). As cell division slows down or ceases, due to reaching stationary growth phase, nutrient starvation, or other stress factors, photosynthetic energy is diverted to lipid production, resulting in the accumulation of TAGs (Hu et al., 2008, Sheehan et al., 1998, Guschina and Harwood, 2006). However, this comes at the cost of decreased growth, which often offsets the higher lipid content, resulting in lower overall productivity (Rodolfi et al., 2009). It is therefore unrealistic to find an algal species that excels in both areas. Fortunately, the complex nature of algal evolution resulted in a wide variety of taxa, with high genetic diversity (Gimpel et al., 2013). This genetic diversity leaves room for improvement through appropriate species selection. Interestingly, species belonging to Stramenopiles and Haptophyta tend to have lower biomass productivity and higher lipid content, while species belonging to Chlorophyta and Rhodophyta are at the opposite end of the productivity spectrum (Fig. 1.1).

It is worthy to note that the Stramenopiles and Haptophyta contain a plastid derived from a secondary endosymbiotic event, while Chlorophyta and Rhodophyta derived their plastid through a primary endosymbiotic event. In addition, a review of 74 published microalgal FA-analysis profiles showed that the fatty acid composition is highly species dependent (Volkman *et al.* 1989, Volkman *et al.* 1991, Viso & Marty 1993, Renaud *et al.* 1999, Tonon *et al.* 2002, Martínez-Fernández *et al.* 2006, Natrah *et al.* 2007, Xu *et al.* 2008). FAs commonly found in algae range from the unsaturated FAs myristic acid (14:0) and palmitic acid (16:0), through the monounsaturated FAs palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9), to the polyunsaturated FAs eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).

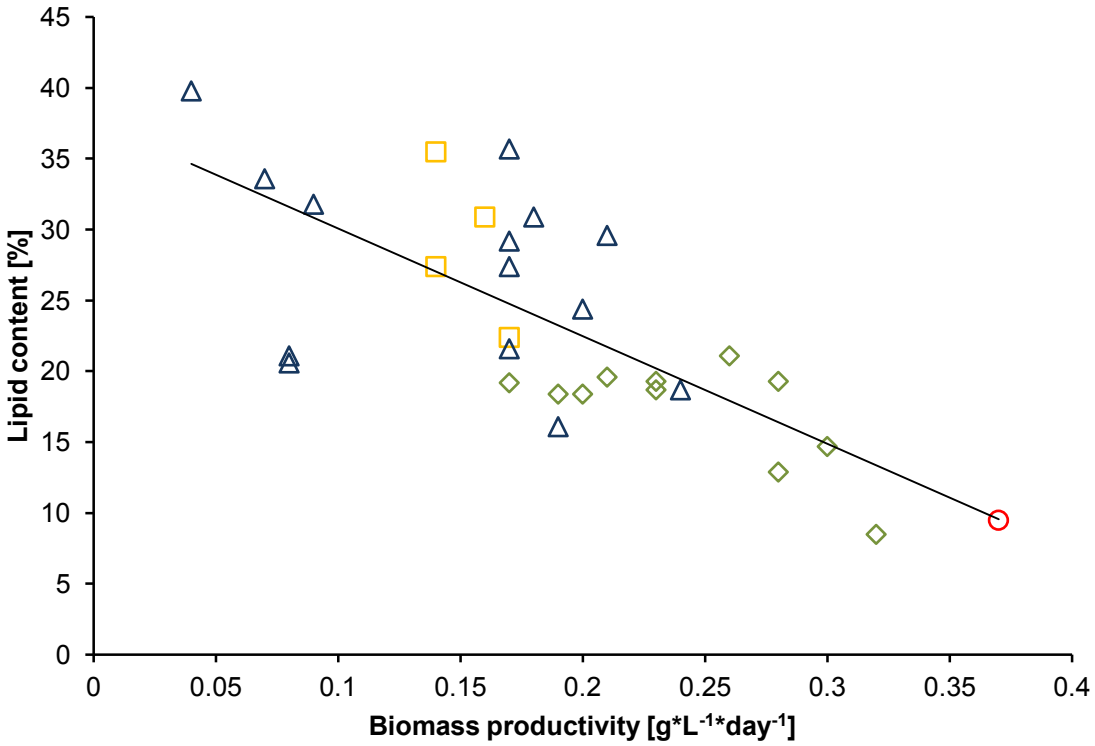

Fig. 1.1 Biomass productivity of different species belonging to the Chlorophyta (green diamonds), Haptophyta (yellow squares), Stramenopiles (blue triangles) and Rhodophyta (red circle) versus lipid content, showing a decrease in lipid content with increasing biomass productivity ($R^2 = 0.5427$) (Based on data taken from Rodolfi et al. (2009))

It becomes apparent that there are distinct differences between the diverse algal species in terms of lipid productivity and FA composition. Since the biochemical pathways in algae are assumed to be conserved, differences in lipid productivity and FA composition are partially based on differences in gene expression patterns. It is therefore important to have a good understanding of algal phylogeny and taxa specific differences in biochemical pathways and gene expression, to facilitate species selection for the different applications (Knothe, 2005).

10. Thesis aims and structure

The overarching goal of this thesis was to increase our knowledge and understanding of microalgal lipid biosynthesis in terms of phylogeny and gene expression patterns. Derived from this goal, the four aims of this thesis were: 1) to describe the fatty acid synthesis pathways in algae with a special focus on ACCase, 2) to investigate the phylogeny of homomeric ACCase and how it relates to current algal taxonomy, 3) to investigate changes in ACCase expression under nitrogen starvation and 4) to investigate changes in desaturase expression under nitrogen starvation. These four aims relate to four data chapters, outlined below. The last chapter comprises of a general synthesis and discussion of all topics covered in this thesis.

Chapter 2 provides the background on algal FA synthesis, with a focus on the key enzyme acetyl-CoA carboxylase (ACCase). It describes the *de novo* FA synthesis pathway, followed by the pathways leading to triacylglycerides, phospholipids, and galactolipids. Furthermore, this chapter describes the different types (heteromeric and homomeric) and locations (cytosolic and plastidial) of ACCase and addresses the presence of either type in the plastid in terms of algal phylogeny.

Chapter 3 describes the phylogeny of the newly sequenced plastidial and cytosolic homomeric ACCases from the algae *Nannochloropsis oculata*, *Isochrysis* aff. *galbana* (TISO) and *Chromera velia*, compared to existing sequences from a variety of other algal species and plants. This phylogeny is discussed in view of past and current opinions on algal taxonomy. In addition, the phylogeny of the plastid-encoded β-carboxyltransferase is investigated.

In chapter 4, the expression of the ACCase genes of *I.* aff. *galbana* (TISO) and *C. velia*, sequenced in Chapter 3, are investigated. Cultures were grown in nitrogen-replete and nitrogen-deplete conditions and samples were harvested during the logarithmic, late logarithmic and stationary growth phases. Gene expression data is correlated to lipid content data.

Chapter 5 reports on the expression of four important fatty acid desaturases in *I.* aff. *galbana* (TISO). Samples from the same cultures grown for chapter 4 were further analysed for this chapter. Gene expression data of the four desaturases is correlated to FA composition data.

Lastly, chapter 6 presents a synopsis of the major research findings described in each of the previous chapters, outlines how the research presented in this thesis overall contributes to our understanding of microalgal lipid biosynthesis, and offers an outlook into further research.

Though research presented in this thesis is entirely my own, publications resulting from chapters 2 to 5 will be, or have been, published as co-authored manuscripts and a "statement of contribution" is given outlining the involvement of the co-authors.

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Chapter 2 - Comprehensive guide to acetyl-CoA carboxylases in algae

The following chapter is a collaborative effort of which each author's contribution is outlined below.

Contribution:
Roger Huerlimann: Conception and execution of project, writing and editing chapter.
Kirsten Heimann: Supervision, editing.

Published: Huerlimann, R. and Heimann, K. (2012). "Comprehensive guide to acetyl-carboxylases in algae." Critical Reviews in Biotechnology **33**(1): 49-65

1. Abstract

Lipids from microalgae have become an important commodity in the last 20 years. Biodiesel and supplementing human diets with ω-3 fatty acids are just two of the many applications. Acetyl-CoA carboxylase (ACCase) is a key enzyme in the lipid synthesis pathway. In general, ACCases consist of four functional domains: the biotin carboxylase (BC), the biotin carboxyl binding protein (BCCP), and α-and ß-carboxyltransferases (α-and ß-CT). In algae, like in plants, lipid synthesis is another function of the plastid. Despite being well researched in plants and animals, there is a distinct lack of information about this enzyme in the taxonomically diverse algae. In plastid-containing organism, ACCases are present in the cytosol and the plastid and two different forms exist, the heteromeric (prokaryotic) and homomeric (eukaryotic) form. Despite recognition of the existence of the two ACCase forms, generalised published statements still list the heteromeric form as the one present in algal plastids. Here it was show this is not the case for all algae. The presence of heteromeric or homomeric ACCase is dependent on the origin of the plastid. ACCase amino acid sequences were compared to show that green and red algae, with the exception of the green algal class Prasinophyceae, contain heteromeric ACCase in their plastids, which are of primary symbiotic origin and surrounded by two envelope membranes. In contrast, algae with plastids surrounded by three to four membranes which were derived through secondary endosymbiosis (Stramenopiles and Haptophyta), as well as apicoplast containing Apicomplexa, contain homomeric ACCase in their plastids. We discovered distinctive differences in the substrate binding regions of heteromeric and homomeric α-CT and β-CT, which can be used to distinguish between the two ACCase types. Furthermore, the acetyl-CoA binding region of homomeric α-CT can be used to distinguish between cytosolic and plastidial ACCase. The information provided here will be of fundamental importance in ACCase expression and activity research in order to unravel impacts of environmental and physicochemical parameters on lipid content and productivity.

2. Introduction

2.1 Why the interest in microalgal lipids?

Lipids from microalgae gained increased interest over the last 20 years because of a wide range of possible applications. Lipids, especially ω-3 fatty acids (FAs), play a very important role as a fish oil replacement in aquaculture feeds (Brown et al., 1997, Martínez-Fernández et al., 2006) and human health foods (reviewed in Pulz and Gross (2004)). More recent applications are biodiesel (Chisti, 2007, Hu et al., 2008, Schenk et al., 2008) and bio-plastics (Ono and Cuello, 2006).

Ideally, due to the high costs associated with microalgal large-scale culturing and harvesting, all parts of the microalgal cell should be converted into products. Other valuable products are colouring substances (pigments: e.g. carotenoids), antioxidants (e.g. β-carotene, astaxanthin), poly-unsaturated fatty acids (PUFAs), and cosmetics (proteins, polysaccharides and lipids) (reviewed in Spolaore et al. (2006)). The remaining biomass after lipid extraction can be used as animal feed additives, fertiliser (reviewed in Pulz and Gross (2004) and Spolaore et al. (2006)), or biochar (Grierson et al., 2009).

Next to proteins and carbohydrates, lipids are one of the three major classes of metabolites in all cells. Lipids are critical to cell function, including energy storage, cell membrane structure and fluidity, and as signalling molecules (Hannun and Obeid, 2008, Moreau et al., 1998). The complete lipid synthesis pathway contains many enzymes in different cellular compartments. Acetyl-CoA carboxylase (ACCase) is located at the start of the lipid synthesis pathway. It is a key enzyme that diverts energy in the form of ATP and carbon in the form of carbon dioxide into the production of fatty acids (FAs) through the production of malonyl-CoA from acetyl-CoA. FAs are the building blocks for many structural (e.g. sphingo-, galacto- and phospholipids) and all storage lipids (e.g. triacylclycerides).

Applications that are based on lipids mainly depend on neutral lipids, in the form of triacylglycerides (TAGs), which are used for energy storage and have no structural function (Hu et al., 2008, Harwood and Jones, 1989). Other glycerol-based lipids are associated with the cell membrane and consist of polar lipids such as glycosylglycerides and phosphoglycerides (Hu et al., 2008).

As a key enzyme, the critical role of ACCase in lipid synthesis is a prerequisite to improve the production of microalgal lipid-derived bio-products (fuel, omega-3 fatty acids etc.). Furthermore, due to the occurrence of two different types of ACCase in the

chloroplast of different algal taxa, it provides a unique opportunity to examine the evolution of plastids in plants and algae.

This chapter will provide a brief overview of the lipid synthesis pathway, followed by a description of the different types of ACCase. The theory of serial endosymbiosis is then introduced, which is important to understand the presence of the two plastidial types of ACCase in different algal taxa. ACCase amino acid sequence comparisons of the different binding regions are used to identify the intracellular location of ACCases and the form present in the chloroplasts of algae and apicoplasts of apicomplexans (collectively referred to as plastids). This information is then related to algal phylogeny. The chapter ends in discussing the impact of ACCase overexpression on lipid yield.

2.2 Brief overview of lipid synthesis pathways

For efficient functioning of the cell and regulatory purposes, biochemical pathways are compartmentalised into various locations within a cell. To understand the importance of different enzymes and their effect on lipid synthesis, it is necessary to have a basic knowledge of the different locations of lipid synthesis. Regardless of the eukaryotic organism (plants, algae, fungi or animals), there is a common basic biosynthetic pathway for the synthesis of FAs and lipids with similar key enzymes being used.

The synthesis of TAGs in eukaryotes can be divided into three major steps: 1) the synthesis of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACCase, EC 6.4.1.2); 2) the elongation of the acyl chain by fatty acid synthase (FAS, EC 2.3.1.85); and 3) the formation of TAGs. However, the location of the different steps varies among organisms.

In organisms that do not contain plastids (such as animals, fungi and bacteria), the first two major steps of lipid synthesis, the de novo synthesis of FA up to C16 or C18 acetyl-CoA, occur in the cytosol (Wolfgang and Lane, 2006). In animal cells, mitochondria supply the acetyl-CoA used in this reaction. Conversely, in plastid-containing organisms (including plants, algae and apicoplasts-containing apicomplexan parasites) the first two major steps of lipid synthesis occur in the plastid, due to the immediate availability of the starter component acetyl-CoA. In photosynthesising plant cells, acetyl-CoA used for the production of malonyl-CoA originates from 3-phosphoglycerate in the Benson-Calvin, through the intermediates 2-phosphoglycerate and phosphoenolpyruvate, ending in the decarboxylation of the pyruvate to acetyl-CoA by the plastid pyruvate dehydrogenase complex (plPDC) (Joyard et al., 2010) (Fig. 2.1). The third step of lipid synthesis, the formation of TAGs, as well as the elongation

(beyond C16 or C18) and desaturation of FAs, are associated within the smooth endoplasmic reticulum (ER) in all eukaryotes (Fig. 2.1).

The lipid synthesis pathway in algae, which occurs in plastids and the smooth ER membrane, is poorly researched compared to animals and plants, but is considered to be similar to plants. In plants, the first two steps are located in plastids, while the last step is located in the smooth ER. However, recent research showed that the green alga *Chlamydomonas reinhardtii* has the ability to assemble TAGs in the plastid (Fan et al., 2011). In plants, ACCase is found in plastids and the cytosol. In plastids, malonyl-CoA is used for *de novo* fatty acid biosynthesis, while malonyl-CoA in the cytosol acts as a precursor for fatty acid elongation and synthesis of secondary metabolites (Nikolau et al., 2003). In animals, on the other hand, *de novo* FA synthesis occurs solely in the cytosol with cytosolic ACCase (ACC1) providing malonyl-CoA (Abu-Elheiga et al., 2000).

FAS produces FAs through condensation of malonyl-ACP with acetyl-ACP (first step of elongation) or acyl-ACP (consecutive steps) (Fig. 2.1). Different acyl-ACP thioesterases (AAT) terminate the chain elongation of FAs through FAS by hydrolysing acyl-ACP into free fatty acids (FFAs) (Joyard et al., 2010). This dictates the chain-length of the newly synthesised FAs, but has most likely no large impact on the FA synthesis rate. As the FAs leave the chloroplast, acetyl-CoA synthase (ACS) joins the FA with a cofactor A (CoA) to form acyl-CoA.

FAs of the acyl-CoA pool in the cytosol can be elongated and desaturated in the smooth ER (Fig. 2.1) (Cook 1996). Furthermore, the production of phospholipids and TAGs also occurs in the smooth ER (Fig. 2.1). One FA is transferred from acyl-CoA to the sn-1 position of glycerol-3-phosphate by acyl-CoA:glycerol-3-phosphate acyl-transferase (GPAT) to form lysophosphatidate (LPA). Lysophosphatidate acyl-transferase (LPAAT) then converts LPA into phosphatidate (PA) by adding another FA chain to the sn-2 position. Phosphatidic acid phosphatase (PAP) converts phosphatidate into diacylglycerol (DAG) by removing the phosphate at sn-3 position. Acyl-CoA:diacylglycerol acyl-transferase (DGAT) is unique to the TAG biosynthetic pathway and catalyses the formation of TAG from DAG and acyl-CoA (for more details see Ohlrogge and Jaworski (1997) and Joyard et al. (2010)).

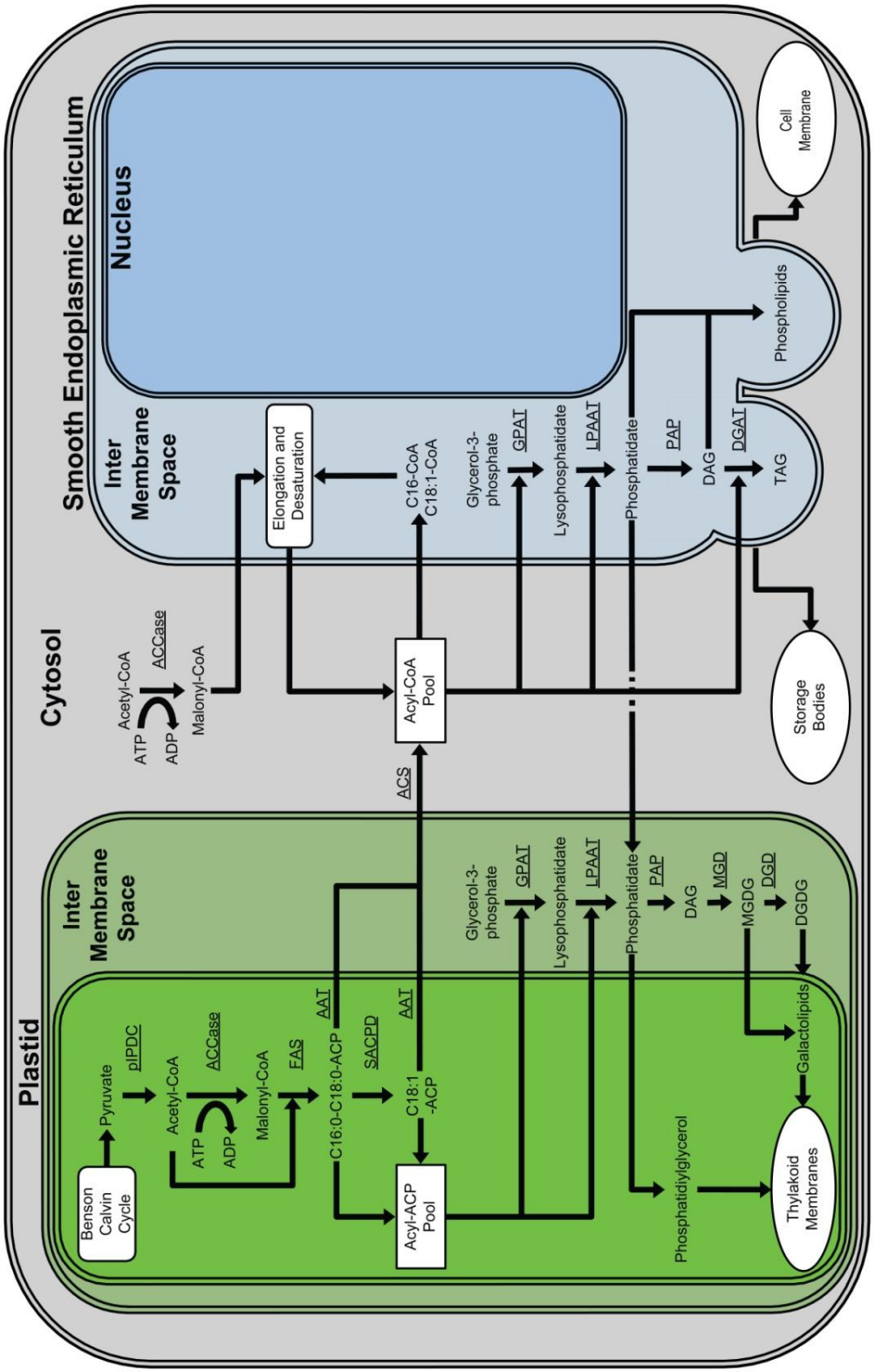

Fig. 2.1 Biochemical pathway of light dependent synthesis of fatty acids and synthesis of triacyl glycerides, phospholipids and galactolipids in plants (Enzymes: *AAT*, acyl-ACP thioesterase; *ACCase*, acetyl-CoA carboxylase;; *ACS*, acyl-CoA synthase; *DGAT*, acyl-CoA:diacylglycerol acyl-transferase; *DGD*, DGDG synthase; *FAS*, type II fatty acid synthase; *GPAT*, acyl-CoA:glycerol-3-phosphate acyl-transferase; *LPAAT*, lysophosphatidate acyl-transferase; *MGD*, MGDG synthase; *PAP*, phosphatidic acid phosphatase; *plPDC*, plastid pyruvate dehydrogenase complex; *SACPD*, stearoyl-ACP desaturase. Substrates and products: *DAG*, Diacylglycerol; *TAG*, Triacylglycerol; *MGDG*, monogalactosyldiacylglycerol; *DGDG*, digalactosyldiacylglycerol. (Illustration based on Ohlrogge and Jaworski (1997), Murphy (1999), Awai et al. (2007), Courchesne et al. (2009), Joyard et al. (2010) and Khozin-Goldberg and Cohen (2011)).

While there are other critical enzymes present in the lipid synthesis pathway, Acetyl-CoA carboxylase occupies a unique position as it is involved in diverting the flow of carbon and energy towards lipid production and away from other biochemical products in the cell (e.g. carbohydrates). However, the structure of ACCase differs significantly among different types of plants and algae and therefore ACCases can be used to determine differences in regulation and activity, as well as phylogenetic problems.

2.3 Structure of ACCase

ACCase occurs in two different forms: 1) a heteromeric (or prokaryotic) form, where the four domains biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and two carboxyltransferases (α-CT and β-CT) are located on individual subunits, and 2) a homomeric (or eukaryotic) form, where the four domains are located on one long multifunctional polypeptide (Fig. 2.2).

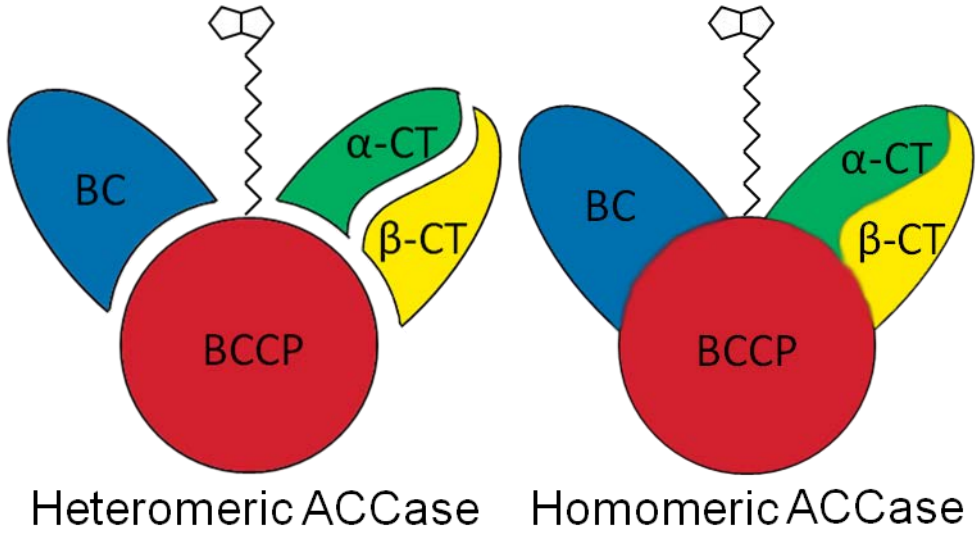

Fig. 2.2 Heteromeric ACCase (prokaryotic) consists of multiple subunits, while homomeric ACCase (eukaryotic) is composed of one long, multifunctional protein (biotin carboxylase (BC), biotin carboxyl carrier (BCC) protein and carboxyltransferase (CT) domain) (Figure modified from Nikolau et al. (2003)).

The heteromeric form is found in prokaryotes and in the plastids of most plants (Table 2.1). The subunits are encoded by four genes, accA (α-CT), accB (BCCP), accC (BC) and accD (β-CT) (Sasaki and Nagano, 2004). In plants, accA, accB and accC are located in the nuclear genome, while accD is plastid-encoded (Sasaki and Nagano, 2004). The high prevalence of accD genes found in the plastidial genomes of algae suggests this is also the case in the algal taxa containing heteromeric ACCase (Supplemental Table 1). However, Chan et al. (2012) found accA, accB and accD encoded in the plastidial genome of *Porphyra* sp., which could indicate that there are differences between Chlorophyta and Rhodophyta. This is supported by the

observation that other plastidial genomes of Rhodophyta analysed for this study often contained sequences for accA, accB and accD, but were lacking sequences of accC, which must be encoded on the nuclear genome (Supplemental Table 1). The homomeric form of ACCase is found in the cytosol of plants, animals, and yeast, as well as in the plastids of Poaceae (true grasses) (Table 2.1). All homomeric ACCases are encoded on the nuclear genome and share a high degree of sequence conservation of the functional domains: NH2-BC-BCCP-CT-COOH (Nikolau et al., 2003). Interestingly, analogous to the structure of ACCase, the type 2 fatty acid synthase (FAS) found in plants, is similar to the FAS found in prokaryotes and consists of a series of different enzymes, while type 1 FAS found fungi and animals consists of one large multifunctional enzyme complex (Joyard et al., 2010).

Table 2.1 Presence or absence of heteromeric and homomeric ACCase in animals, plants, yeast, and bacteria.

Organism	Heteromeric ACCase	Homomeric ACCase	Source
Animals	-	Cytosol/mitochondria	(Brownsey et al., 2006)
Plants (non-Poaceae)	Plastids	Cytosol	(Konishi et al., 1996)
Plants (Poaceae)	-	Cytosol/Plastids	(Konishi et al., 1996)
Yeast	-	Cytosol	(Al-Feel et al., 1992)
Bacteria	Cytosol	-	(Magnuson et al., 1993)
Archaea	Cytosol	-	(Chuakrut et al., 2003)

The main function of the BCCP domain is to provide an attachment point for the biotin molecule (Roessler and Ohlrogge, 1993). BC contains an ATP-binding motif, as well as a CO_2 fixation site (Kimura et al., 2000). Acetyl-CoA binds to a specific region on α-CT, while β-CT contains a binding region for carboxybiotin (Roessler and Ohlrogge, 1993).

The production of malonyl-CoA by ACCase is divided into two partial reactions: 1) the BC domain carboxylates the biotin prosthetic group bound to the structural BCCP domain; 2) the CT domains transfer the carboxyl group to acetyl-coA (Nikolau et al., 2003).

1) $BCCP + HCO_3^- + Mg^{2+}\text{-}ATP \rightarrow BCCP\text{-}CO_2^- + Mg^{2+}\text{-}ADP + Pi$

2) $BCCP\text{-}CO_2^- + \text{acetyl-CoA} \rightarrow BCCP + \text{malonyl-CoA}$

The naming of homomeric ACCases is inconsistent between different kingdoms. In animals, the cytosolic ACCase is labelled ACC1, while ACC2 is located in mitochondria where malonyl-CoA is not used as a substrate, but is involved in the regulation of β-oxidation (Abu-Elheiga et al., 2000). In *Arabidopsis thaliana*, ACC1 is expressed in the cytosol, while ACC2 is targeted at the plastid (Bryant et al., 2011). However, the plastidial homomeric ACCase is expressed at low levels and cannot maintain viability in embryos in which the heteromeric ACCase was disabled (Bryant et al., 2011). In

Cyclotella cryptica (diatom) (Dunahay et al., 1995) and *Toxoplasma gondii* (apicomplexan parasite) (Zuther et al., 1999), on the other hand, ACC1 is located in the chloroplast and ACC2 in the cytosol. In *Chlorella* sp. (green alga), the gene that codes for the cytosolic homomeric ACCase is called *acc*1 (Wan et al., 2011). To avoid confusion we will use ACC1 for plastidial ACCase and ACC2 for cytosolic ACCase when talking about algae.

In algae, there is no clear information on the plastidial form of ACCase even though this is essential to investigate the regulation and expression of this key enzyme in FA synthesis. Riekhof et al. (2005) identified the presence of the heteromeric form of ACCase in chloroplasts of the green alga *Chlamydomonas reinhardtii* (Chlorophyceae) based on similarities with known amino acid sequences from other species and the analysis of the targeting and signalling sequence. In contrast, plastidial ACCase has been reported to be homomeric for the haptophyte *Isochrysis galbana* (Livne and Sukenik, 1990) and the diatom *Cyclotella cryptica* (Roessler, 1990, Roessler and Ohlrogge, 1993). Even though the possible presence of heteromeric ACCase in *C. cryptica* cannot be completely excluded, no heteromeric ACCase was found (Roessler, 1990). Furthermore, ACCase from *C. cryptica* contained a specific signal peptide (Roessler and Ohlrogge, 1993), which exhibits similarities to the signal peptide targeting proteins to the chloroplast ER in the diatom *Phaeodactylum tricornutum* (Bhaya and Grossman, 1991).

Even though the presence of the different forms of ACCase has been thoroughly investigated in plants and animals, there has been no thorough review on which form of ACCase is present in different algal taxa. First indications on the distribution of the two different ACCase forms can be derived from the recently published plastidial and full transcriptomes/genomes of different algal species. To elucidate the presence and cellular location of heteromeric or homomeric ACCase in four major algal taxa and an apicoplasts-containing apicomplexan, we reviewed the existing literature and published information on ACCase, and combined this with full genome searches of different algal species.

3. The theory of serial endosymbiosis

Like mitochondria, chloroplasts arose from an endosymbiotic event. In the case of primary plastids, a eukaryotic cell engulfed a cyanobacterium, which was reduced to an organelle over time. Interestingly, both mitochondria and chloroplasts are important to the energy supply of cells. Mitochondria are responsible for the conversion of energy by generating ATP through the metabolism of pyruvate, the product of glycolysis of

glucose; conversely, plastids use energy derived from light to build energy-rich biomolecules in the form of glucose and fatty acids. The β-oxidation of FAs occurs in peroxisomes in plants and in mitochondria in animals. While mitochondria, which are common to all eukaryotic cells, arose from one endosymbiotic event, the origin of plastids is more complex and included several different endosymbiotic events.

To fully understand the presence of the two types of ACCase in algal plastids, the evolution of the different algal taxa and the endosymbiotic origin of their chloroplasts need to be comprehended first. The endosymbiotic theory was reviewed by Archibald (2008) and Gould et al. (2008) and is summarised here.

The endosymbiotic theory states that chloroplasts with two envelope membranes, found in Chlorophyta (green algae), Rhodophyta (red algae) and Glaucocystophyta, arose through a primary endosymbiotic event where a heterotrophic eukaryote engulfed a cyanobacterium. This gave rise to photosynthetic eukaryotes containing first generation chloroplasts (Table 2.2, Fig. 2.3, Fig. 2.4). The change from an endosymbiont to an organelle required gene transfer from the genome of the cyanobacterium to the nucleus of the host, but also included the complete loss of certain plastid genes (Fig. 2.5).

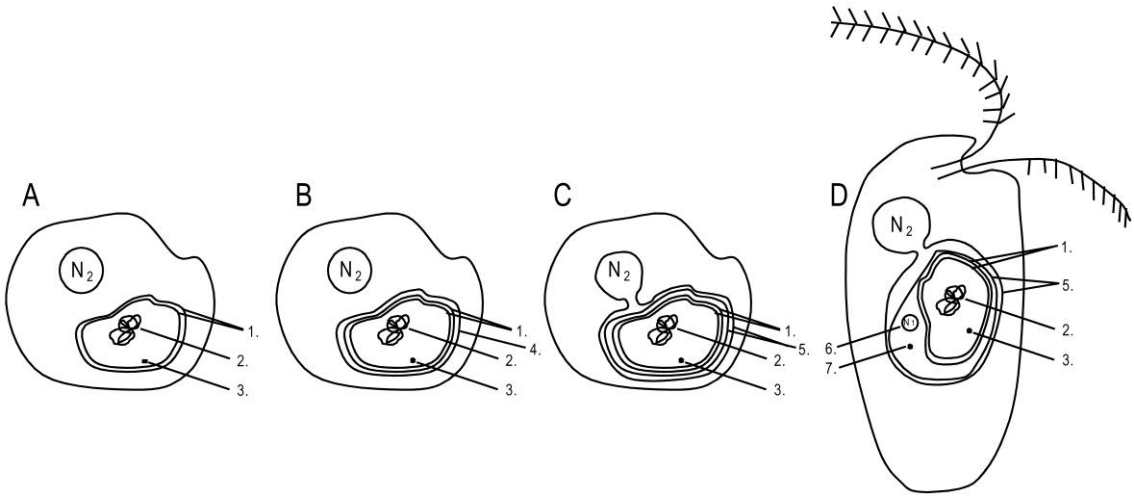

Fig. 2.3 A) algae with first generation chloroplast, B) algae with second generation chloroplast with 3 membranes, C) algae with second generation chloroplast with 4 membranes, two of which form the chloroplast endoplasmic reticulum D) algae with second generation chloroplast with 4 membranes, two of which form the chloroplast endoplasmic reticulum (CER) membrane, including a nucleomorph. 1. Chloroplast envelope derived from primary endosymbiotic event, 2. Bacterial chromosome, 3. Prokaryotic ribosomes, 4 Chloroplast envelope(s) derived from secondary endosymbiotic event, 5. CER outer membrane continuous with nuclear envelope and inner membrane compartmentalising the chloroplast from the ER, 6. Nucleomorph: remnant nucleus N_1 of eukaryotic symbiont (present only in Cryptophyta and Chlorarachniphyta), 7. eukaryotic ribosomes. N2 host nucleus.

Table 2.2 Taxonomic relationship between chloroplasts of plants and different algal taxa, based by primary, secondary and tertiary endosymbiotic events.

Organism	**Cyanobacteria**	**Organisms with 1st generation plastids (primary endosymbiosis)**	**Organisms with 2nd generation plastids (secondary endosymbiosis)**	**Organisms with 2nd or 3rd generation plastids (tertiary endosymbiosis)**
Envelope membranes	None	2 envelope membranes	3-4 envelope membranes	3-4 envelope membranes
Members		Viridiplantae	Chromalveolata	Chromalveolata
		● Plantae (Higher plants)	● Stramenopiles	● Dinoflagellata
		- Eudicots	- Bacillariophyceae (diatoms)	Rhizaria
		- Monodicots	- Phaeophyceae (brown algae)	● Cercozoa
		- Pteridophyta (Ferns)	- Eustigmatophyceae	- Chlorarachniophyta
		- Bryophyta (Mosses)	- Chrysophyceae	
		● Chlorophyta (green algae)	● Haptophyta	
		● Rhodophyta (red algae)	● Cryptophyta	
		● Glaucocystophyta (algae)	Euglenozoa	
			● Euglenoidea	

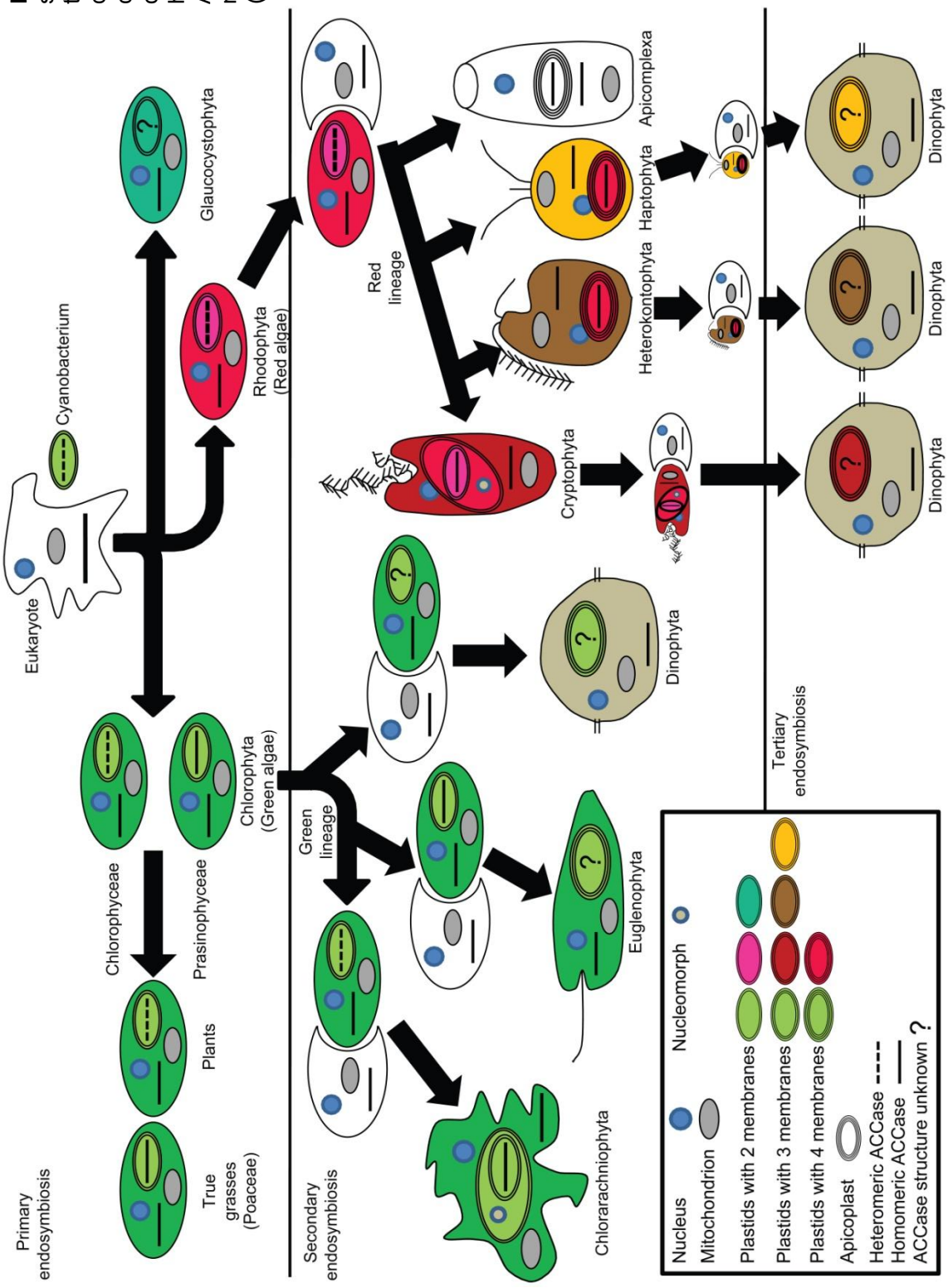

Fig. 2.4 ACCase taxonomy showing first, second and third generation plastids in different algal taxa and the current state of knowledge on the presence of heteromeric or homomeric ACCase in plastids (Figure modified from Wilson (2005)).

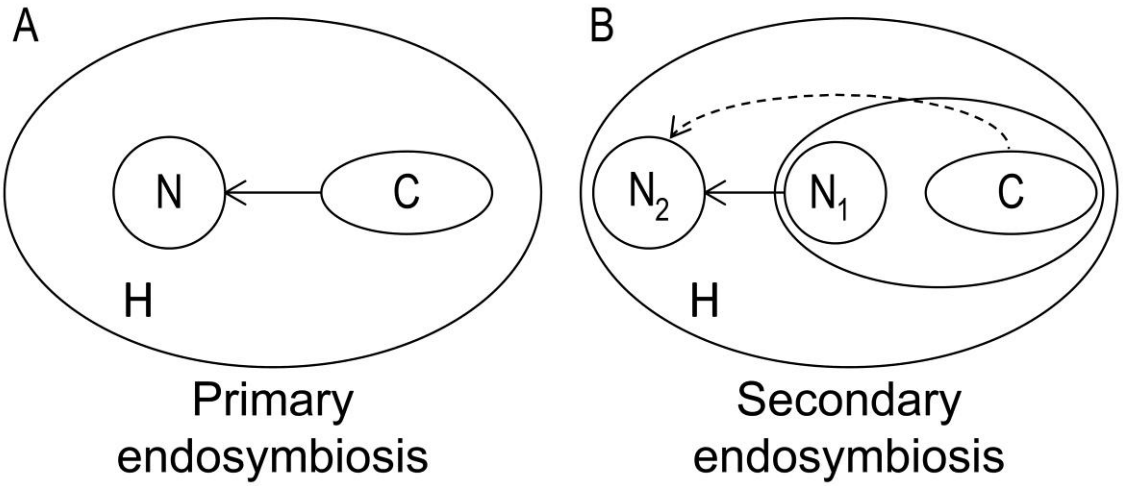

Fig. 2.5 Gene transfer (arrows) from chloroplast (C) to nucleus (N) of host (H) after primary endosymbiotic event A) and from the nucleus of chloroplast (N_1) to host nucleus (N_2) after secondary endosymbiotic event B). The dashed arrow shows the unlikely but possible gene transfer directly from the plastidial genome to the host nucleus (N_2) after the secondary endosymbiotic event.

These first generation photosynthetic eukaryotes in turn were then taken up by other heterotrophic eukaryotes in a secondary endosymbiotic event (Table 2.2, Fig. 2.4). This resulted in second generation plastids after additional gene transfer from the eukaryotic endosymbiont to the nucleus of the new eukaryotic host (Fig. 2.5). Chloroplasts of second and third generation origin contain 3-4 membranes. Dinoflagellates are unique in that it is believed that some taxa took up photosynthetic eukaryotes of diverse taxonomic origin but also lost established plastids again (Howe et al., 2008). Some dinoflagellates even may have lost and taken up new symbiotic relationships again several times in sequence (Howe et al., 2008). Only approximately 50% of the dinoflagellates and euglenoids are photosynthetic; the majority of these have 3 membranes (Fig. 2.4, Fig. 2.3). Haptophytes, cryptophytes, chlorachaniophytes and stramenopiles contain 4 membranes and the outermost membrane is continuous with the rough ER of the secondary host (Fig. 2.3). The outermost two envelope membranes are therefore called the chloroplast ER (cER). Apicomplexans also contain 4 membranes around the plastid (apicoplasts), and even though they are not continuous with the ER, there is a close association between the apicoplast and the ER (Kalanon and McFadden, 2010).

Glaucocystophyta, Cryptophyta and Chlorarachniophyta represent three bridging organisms. The glaucocystophytes form a special case, where the bacterial peptidoglycan wall is still present between the inner and outer envelope membrane of the cyanelle (photosynthetic plastid). The unique feature of cryptophytes and chlorarachniophytes, on the other hand, is the presence of a nucleomorph, which is the

remnant of the nucleus of the photosynthetic eukaryote taken up during the 2nd endosymbiotic event (Fig. 2.3).

4. ACCase structure in algae

4.1 Genomic information on the type of ACCase present in algae

The evolution of algae by primary, secondary and tertiary endosymbiosis of different symbionts by a variety of hosts resulted in a large number of different algal taxa (Fig. 2.4). Concerning the important enzyme ACCase, there has been no review summarising the presence or absence of the homomeric and heteromeric ACCases. The number of full genome sequences is increasing, but at the time of this review, only 17 full genome sequences of algae were publically accessible (Table 3). Chloroplast genome sequences are an additional source to investigate the presence of heteromeric ACCase (Table 3). The presence of the β-CT subunit in chloroplast genomes shows that green algae containing heteromeric ACCase have the same gene distribution as eudicot plants. While the presence of the *acc*D gene coding for β-CT in the chloroplast is no absolute guarantee for the functional expression of heteromeric ACCase, this gene has thus far not been found in algae with homomeric ACCase in the chloroplast.

A desktop study using proteomic data was used to tabulate the presence and location of the respective ACCase types in algae (Table 3, Supplemental Table 1). Complete proteomes of 17 microalgal species from different taxa were obtained from the Joint Genome Institute (University of California, www.jgi.doe.gov, *Aureococcus anophagefferens* (Gobler et al., 2011), *Chlamydomonas reinhardtii,* (Merchant et al., 2007), *Chlorella* sp. (NC64A) (Blanc et al., 2010), *Bigelowiella natans* (Curtis et al., 2012), *Fragilariopsis cylindrus* (Mock et al., unpublished), *Guillardia theta* (Curtis et al., 2012), *Micromonas pusilla* (Worden et al., 2009), *Micromonas* sp. (RCC299) (Worden et al., 2009), *Ostreococcus lucimarinus* (Palenik et al., 2007), *Ostreococcus* sp. (RCC809) (unpublished, genome.jgi-psf.org/OstRCC809_2/OstRCC809_2.home.html), *Phaeodactylum tricornutum* (Bowler et al., 2008), *Thalassiosira pseudonana* (Armbrust et al., 2004), *Volvox carteri* (Prochnik et al., 2010), *Emiliania huxleyi* (unpublished, http://genome.jgi-psf.org/Emihu1/Emihu1.home.html)), an additional one from the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/ (Matsuzaki et al., 2004)) and a further two (*Bathycoccus* sp. (Moreau and Vandepoele, unpublished) and *Ectocarpus siliculosus* (Cock et al., 2010)) from the Bioinformatics Online Genome Annotation System (http://bioinformatics.psb.ugent.be/webtools/bogas/).

Table 2.3 Summary of the presence and location of homomeric and heteromeric ACCase. N/A denotes no information available. (Details and source of the individual sequences are in Supplemental Table 1).

Species (Strain)	Phylum/Division	Class	Heteromeric	Homomeric	
			Plastid[1]	Cytosol	Plastid
Toxoplasma gondii	Apicomplexa	-	Absent	Present	Present
Chaetosphaeridium globosum	Charophyta	-	β-CT	N/A	N/A
Chara vulgaris	Charophyta	-	β-CT	N/A	N/A
Chlorokybus atmophyticus	Charophyta	-	β-CT	N/A	N/A
Staurastrum punctulatum	Charophyta	-	β-CT	N/A	N/A
Zygnema circumcarinatum	Charophyta	-	β-CT	N/A	N/A
Bigelowiella natans	Chlorarachniophyta	-	Absent	Present	Present
Auxenochlorella protothecoides	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Helicosporidium sp.	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Leptosira terrestris	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Bryopsis hypnoides	Chlorophyta	Bryopsidophyceae	β-CT	N/A	N/A
Chlamydomonas reinhardtii	Chlorophyta	Chlorophyceae	BC, BCCP1, BCCP2, α-CT, β-CT	N/A	N/A
Chlorococcum humicola	Chlorophyta	Chlorophyceae	β-CT	N/A	N/A
Dunaliella salina	Chlorophyta	Chlorophyceae	β-CT	N/A	N/A
Haematococcus pluvialis	Chlorophyta	Chlorophyceae	BC	N/A	N/A
Polytomella parva	Chlorophyta	Chlorophyceae	BCCP	N/A	N/A
Volvox carteri f. *nagariensis*	Chlorophyta	Chlorophyceae	BC, BCCP1, BCCP2, α-CT, β-CT	Present	-
Pedinomonas minor	Chlorophyta	Pedinophyceae	β-CT	N/A	N/A
Bathycoccus prasinos	Chlorophyta	Prasinophyceae	Absent	Present	Present
Micromonas pusilla	Chlorophyta	Prasinophyceae	Absent	Present	Present
Micromonas sp.	Chlorophyta	Prasinophyceae	Absent	Present	Present
Nephroselmis olivacea	Chlorophyta	Prasinophyceae	β-CT	N/A	N/A
Ostreococcus lucimarinus	Chlorophyta	Prasinophyceae	Absent	Present	Present
Ostreococcus sp.	Chlorophyta	Prasinophyceae	Absent	Present	Present
Chlorella sp.	Chlorophyta	Trebouxiophyceae	BC, BCCP1, BCCP2, α-CT	Present	Absent
Coccomyxa sp.	Chlorophyta	Trebouxiophyceae	BC, BCCP, α-CT, β-CT	N/A	N/A
Muriella zofingiensis	Chlorophyta	Trebouxiophyceae	BC	N/A	N/A
Oocystis solitaria	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Parachlorella kessleri	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Trebouxia aggregata	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Trebouxiophyceae sp.	Chlorophyta	Trebouxiophyceae	β-CT	N/A	N/A
Oltmannsiellopsis viridis	Chlorophyta	Ulvophyceae	β-CT	N/A	N/A
Pseudendoclonium akinetum	Chlorophyta	Ulvophyceae	β-CT	N/A	N/A
Guillardia theta	Cryptophyta	-	Absent	Present	Present
Microcystis aeruginosa	Cyanobacteria	Chroococcales	BC, BCCP, α-CT, β-CT	Absent	Absent
Anabaena variabilis	Cyanobacteria	Nostocales	BC, BCCP, α-CT, β-CT	Absent	Absent
Nostoc azollae	Cyanobacteria	Nostocales	BC, BCCP, α-CT, β-CT	Absent	Absent
Emiliana huxleyi	Haptophyta	-	Absent	Present	Present
Isochrysis galbana	Haptophyta	-	Absent	N/A	Present
Antithamnion sp.	Rhodophyta	-	α-CT	N/A	N/A
Cyanidioschyzon merolae	Rhodophyta	-	BC, BCCP, α-CT, β-CT	Present	Absent
Cyanidium caldarium	Rhodophyta	-	BCCP, α-CT, β-CT	N/A	N/A
Galdieria sulphuraria	Rhodophyta	-	BC, BCCP, α-CT, β-CT	Present	Absent
Gracilaria tenuistipitata var. liui	Rhodophyta	-	BCCP, α-CT, β-CT	N/A	N/A

Species (Strain)	Phylum/Division	Class	Heteromeric	Homomeric	
			Plastid[1]	Cytosol	Plastid
Porphyra purpurea	Rhodophyta	-	BCCP, α-CT, β-CT	N/A	N/A
Porphyra umbilicalis	Rhodophyta	-	BCCP, α-CT, β-CT	N/A	N/A
Porphyra yezoensis	Rhodophyta	-	BCCP, α-CT, β-CT	N/A	N/A
Cyclotella cryptica	Stramenopile	Bacillariophyceae	Absent	Present	Present
Fragilariopsis cylindrus	Stramenopile	Bacillariophyceae	Absent	Present	Present
Phaeodactylum tricornutum	Stramenopile	Bacillariophyceae	Absent	Present	Present
Thalassiosira pseudonana	Stramenopile	Bacillariophyceae	Absent	Present	Present
Nannochloroposis gaditana	Stramenopile	Eustigmatophycea	Absent	N/A	Present
Aureococcus anophagefferens	Stramenopile	Pelagophyceae	Absent	Present	Present
Ectocarpus siliculosus	Stramenopile	Phaeophyceae	Absent	Present	Present

[1] Cytosol for bacteria

These complete proteomes were used to generate individual databases in BioEdit (Hall, 2001), which were then searched with known amino acid (aa) sequences of homomeric ACCase and the four subunits of heteromeric ACCase from *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, *Cyclotella cryptica* and *Escherichia coli* obtained from GeneBank (Benson et al., 2009) (Supplemental Table 2), using a BLAST algorithm (Altschul et al., 1997). The resulting aa sequences with strong similarities to the known aa sequences where then manually annotated by BLASTing them on NCBI (www.ncbi.nlm.nih.gov), using existing annotation, comparing length and the presence of active regions based on Roessler and Ohlrogge (1993). To complete the dataset, additional available sequences were obtained from NCBI (Supplemental Table 1). To interpret this dataset, it is important to include the taxonomic relationships of algae, based on the endosymbiotic origin of plastids as outlined in section 3.

4.2 Synthesis of the endosymbiotic theory and ACCase type in algae

Combining the proteomic findings with information on algal taxonomy and the endosymbiotic theory a clear trend emerges. All investigated algae with first generation plastids belonging to the red algae (Rhodophyta) and the green algal (Chlorophyta) class Chlorophyceae contain heteromeric ACCase in their chloroplasts, while the ACCase type present in organelles of glaucocystophytes is still unknown (Table 3, Fig. 2.4). An exception to the above is the green algal class Prasinophyceae, which contain homomeric ACCase in their plastids (Table 3, Fig. 2.4). BC and α-CT were found in the proteome of the prasinophyte *Bathyococcus*, in addition to two homomeric ACCases. However, closer inspection showed that these two sequences most likely are bacterial contamination. On the other hand, none of the investigated algal taxa with second generation chloroplasts contained heteromeric ACCase (Table 3, Fig. 2.4).

No complete proteomes or other published information on the type of ACCase present in dinoflagellates was found. However, inferences can be made by investigating apicomplexan parasites. Apicomplexan parasites are closely related to dinoflagellates, and together they are grouped into the superphylum Alveolata. The newly discovered photosynthetic microalgae *Chromera velia* of the phylum Chromerida has been shown to be the missing link between apicomplexans and dinoflagellates, exhibiting features of both (Moore et al., 2008). Apicomplexan parasites contain a chloroplast relict, called apicoplast, which lacks pigments and no longer carries out photosynthesis, but is entirely devoted to lipid synthesis (Moore et al., 2008). Like algae with second generation plastids, the apicoplast of the apicomplexan parasite *Toxoplasma gondii* also contained homomeric ACCase (Zuther et al., 1999). Furthermore, the homomeric ACCase found in the apicoplasts of *T. gondii* shows a large degree of sequence similarity to the homomeric ACCase found in the chloroplasts of the diatom *Cyclotella cryptica* (Zuther et al., 1999).

4.3 Sequence alignment of binding regions of ACCase

Using the information of the collected sequences (Supplemental Table 1), the respective binding domains of BC, BCCP and α-CT and β-CT of heteromeric and homomeric ACCase are being compared between selected sequences of different species and phyla. Binding domains are highly conserved and serve as a good basis for a comparison and identification of sequences (Kimura et al., 2000, Roessler and Ohlrogge, 1993). Sequences where binding domains could not be found were excluded from the comparison.

Complete sequences, including signalling peptides, may not always be available. Being able to distinguish between cytosolic and plastidial, as well as heteromeric and homomeric ACCase from partial fragments may be achieved by analysing aa sequences of the different binding regions, as discussed below. The aa G at position 7 of the α-CT binding region has been conserved between the two forms of ACCase, but aa sequences at positions 1 to 3 reveal there is a clear difference between the heteromeric α-CT subunit and the homomeric α-CT domain (Fig. 2.6). The following unifying characteristics can be derived from the observations outlined below. Species with second generation endosymbionts contain homomeric ACCase in the plastids with the acetyl-CoA binding region of the homomeric α-CT domain starting with the motif GKS. All cytosolic homomeric ACCases, irrespective of the origin of the plastid, start with an A, except for *A. anophagefferens*, *Guillardia theta* and five species belonging to the green algal class Prasinophycae, which start with a G instead.

Out of the 37 homomeric sequences, 17 start with the motif GKS (sequences in green, Fig. 2.6). This motif is always present in homomeric ACCases of algae that have been positively identified to be targeted to the chloroplast (algal species in green, ACC1 of *Cyclotella cryptica* and *Toxoplasma gondii*). It also appears to be applicable to other sequences, which are therefore presumed to be targeted to the chloroplast. Thirteen regions start with the aa A, while the second and third aa exhibit some variation (sequences in blue, Fig. 2.6). Of these 13 regions, the sequences of all five species which are known to only contain cytosolic ACCase (species in blue: yeast, rat, ACC2 of *Toxoplasma gondii* and ACC1 of *Arabidopsis thaliana*) start with an A (Fig. 2.6). The plastidial ACCase of *A. thaliana* (ACC2) also starts with an A. This is an indication that the plastidial ACCase in plants is of a different origin than the plastidial ACCase in algae, which will be discussed further in chapter 3. The remaining seven sequences belong to the green algal class Prasinophyceae (*Ostreococcus*, *Micromonas* and *Bathyococcus*), the Cryptophyta (*Guillardia theta*) and the Stramenopile class Pelagophyceae (*Aureococcus anophagefferens*). They all contain two homomeric ACCases. One region starts with the normal GSK motif as predicted, presumably identifying it as plastidial ACCase. However, in the second region the starting A seems to have been replaced with a G (yellow, Fig. 2.6).

As with the acetyl-CoA binding region, the heteromeric β-CT subunit and homomeric β-CT domain exhibit distinct differences and conserved sequences in the carboxybiotin binding domain (Fig. 2.7 and Fig. 2.8). The most distinctive feature is the conserved motif GAR at the end of all sequences and a G in position 16 (Fig. 2.7). There are no distinctive features that could be used to distinguish between the cytosolic and plastidial form of the homomeric ACCase (Fig. 2.7). The sequences of the binding region of the heteromeric β -CT subunit show a higher homology with each other, compared to the sequences within the homomeric β -CT domain, especially the presence of a cassette from aa 13 to 25, which is highly conserved within the heteromeric form, but not found in the homomeric form (Fig. 2.7). However, all homomeric β-CT domain carboxylbiotin binding regions start with GR and the third aa is variable, while no strictly conserved aa sequence is observable at the start of the binding domain of the heteromeric β-CT subunit (Fig. 2.7).

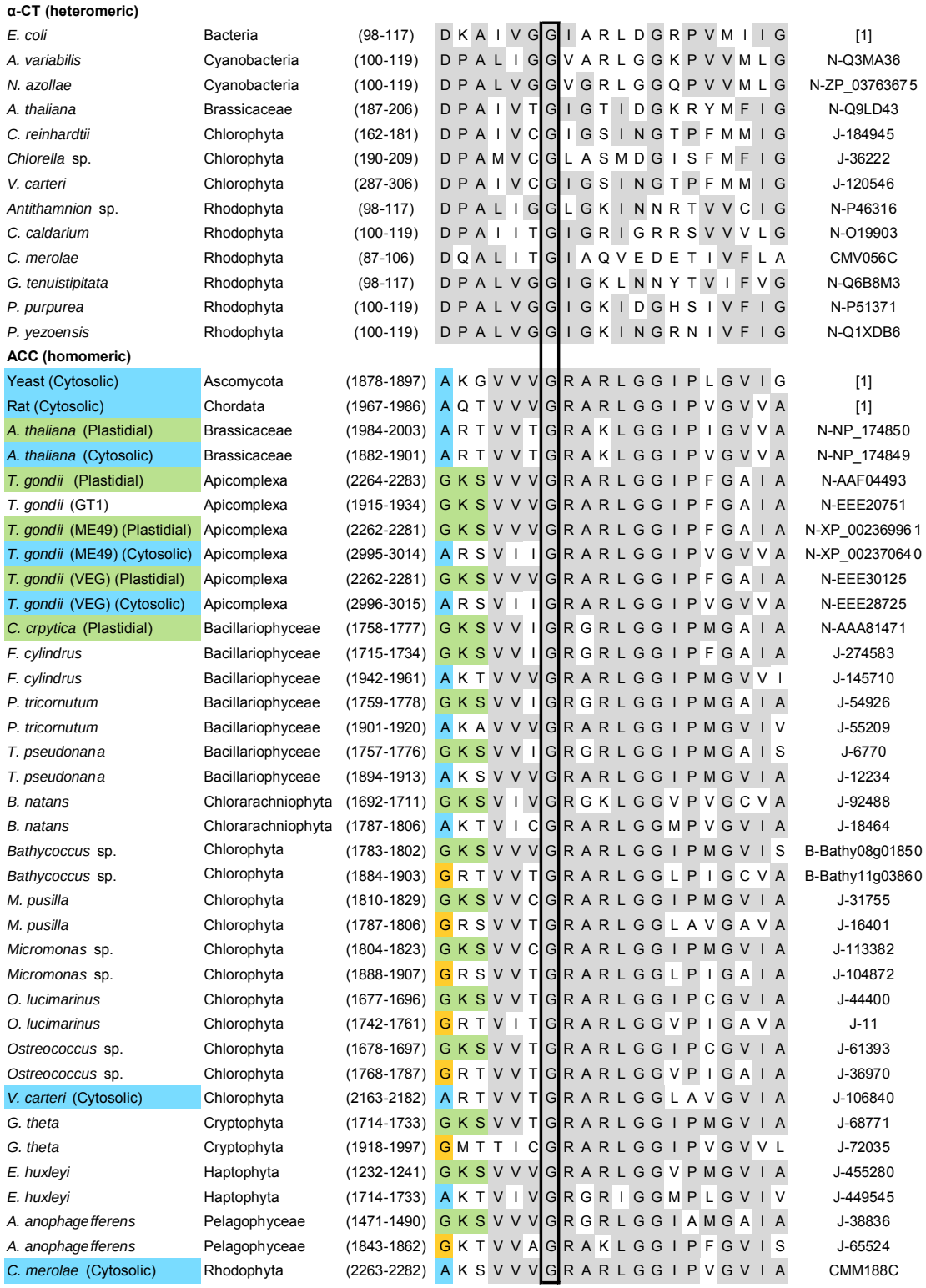

Fig. 2.6 Acetyl-CoA binding region of α-carboxyl transferase (Roessler and Ohlrogge, 1993). Grey highlights the most conservative regions within the heteromeric and homomeric forms. Blue and green species names denote homomeric ACCase positively identified to be found in the cytosol and chloroplast, respectively. Blue and green coloured amino acids highlight different motifs that can be used to distinguish between cytosolic and chloroplastic ACCase. Yellow amino acids denote motifs that deviate from the general hypothesis. Solid lines identify conserved regions in both heteromeric and homomeric ACCase. Details on genera can be found in Supplementary Material 1. (References: [1] Roessler and Ohlrogge (1993), J: Joint Genome Institute, N: NCBI, B: BOGAS, CM: *Cyanidioschyzon merolae* Genome Project).

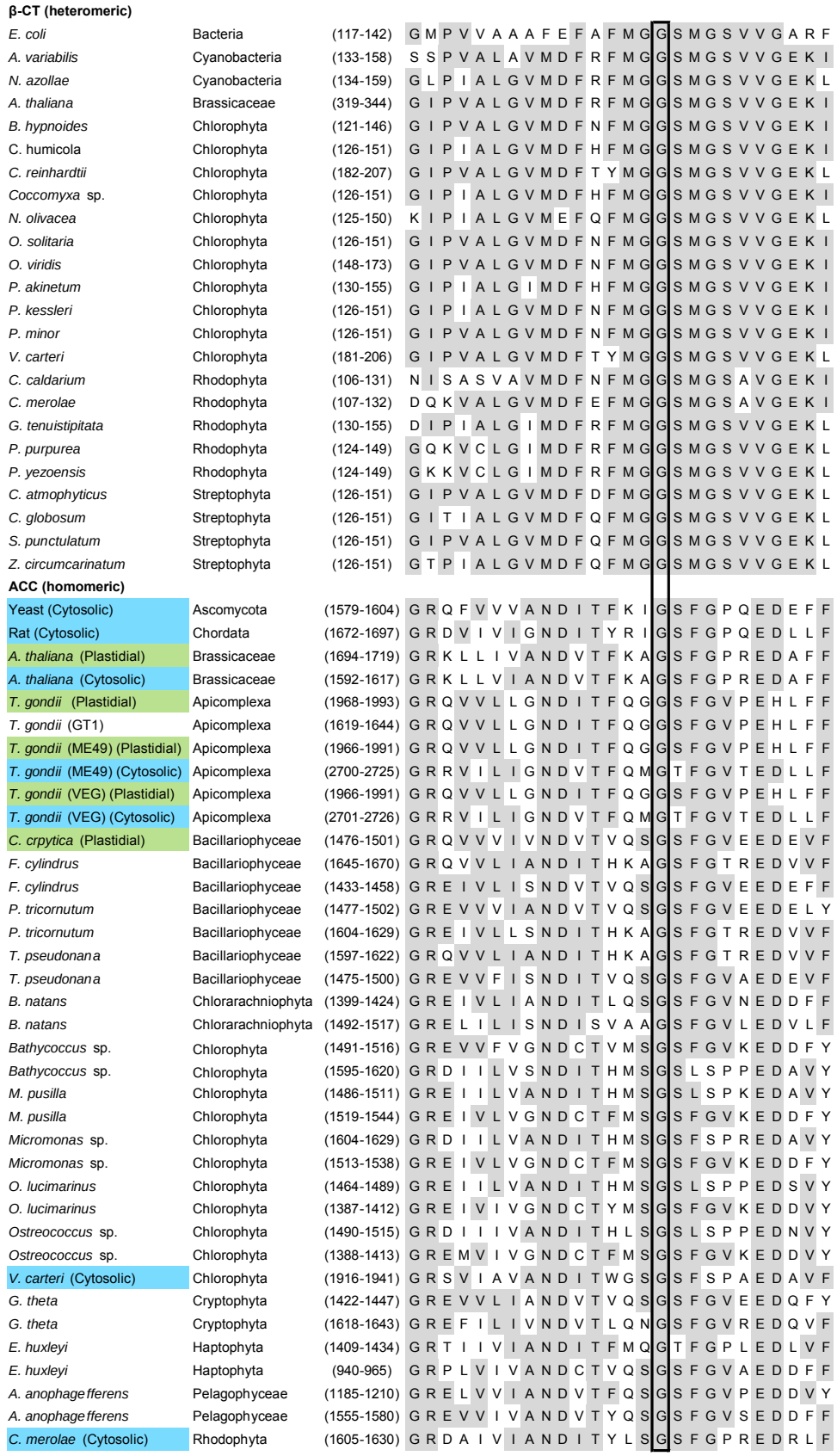

Fig. 2.7 First 26 amino acids of the 51 amino acid carboxylbiotin binding region of β-carboxyl transferase (Roessler and Ohlrogge 1993). Grey highlights the most conservative regions within the heteromeric and homomeric forms. Blue and green species names denote homomeric ACCase positively identified to be found in the cytosol and chloroplast, respectively. Solid lines identify conserved regions in both heteromeric and homomeric ACCase. Details on genera can be found in Supplementary Material 1. (References: [1] Roessler and Ohlrogge (1993), J: Joint Genome Institute, N: NCBI, B: BOGAS, CM: *Cyanidioschyzon merolae* Genome Project).

β-CT (heteromeric)				
E. coli	Bacteria	(143-167)	V R A V E Q A L E D N C P L I C F S A S G G A R M	[1]
A. variabilis	Cyanobacteria	(159-183)	T R L I E Q A T Q R R Y P V V I I C T S G G A R M	N-YP_320706
N. azollae	Cyanobacteria	(160-184)	T R L I E Q A T Q R R Y P V V I I C T S G G A R M	N-ZP_03766652
A. thaliana	Brassicaceae	(345-369)	T R L I E Y A T N Q C L P L I L V C S S G G A R M	N-P56765
B. hypnoides	Chlorophyta	(147-171)	V R L V E Y A T Q E G L I L I L I C A S G G A R M	N-YP_003227083
C. humicola	Chlorophyta	(152-176)	T R L I E Y A T Q E G L P V I L V C A S G G A R M	N-ADZ15145
C. reinhardtii	Chlorophyta	(208-232)	T R L I E Y A T Q E G M P V I I V C T S G G A R M	J-133238
Coccomyxa sp.	Chlorophyta	(152-176)	T R L I E Y A A Q E G L T L V L V C A S G G A R M	N-YP_004222027
N. olivacea	Chlorophyta	(151-175)	T R L I E S A T I S G I A I V I V C A S G G A R M	N-NP_050834
O. solitaria	Chlorophyta	(152-176)	T R L I E Y A T Q E G L T L I L I C A S G G A R M	N-ACQ90742
O. viridis	Chlorophyta	(174-198)	T R L I E Y A T V E G L T L L I V C A S G G A R M	N-YP_635822
P. akinetum	Chlorophyta	(156-180)	T R L I E Y A T K K G L T L I I I S A S G G A R M	N-YP_636254
P. kessleri	Chlorophyta	(152-176)	T R L I E Y A T Q E G L T L I L V C A S G G A R M	N-YP_003058286
P. minor	Chlorophyta	(152-176)	T R L I E Y A T Q E G L T L I L V C A S G G A R M	N-ACQ90825
V. carteri	Chlorophyta	(207-231)	T R L I E Y A T Q E G M P V I I V C T S G G A R M	J-82311
C. caldarium	Rhodophyta	(132-156)	T R L V E F S T K E E L P I V I I S A S G G A R M	N-Q9TLW3
C. merolae	Rhodophyta	(133-157)	T R L I E T S I S Q A L P L I L I S A S G G A R M	CMV207C
G. tenuistipitata	Rhodophyta	(156-180)	T R L I E K A T I E K I P V L I I C A S G G A R M	N-YP_063517
P. purpurea	Rhodophyta	(150-174)	T R L L E K A T Q E R L P A I I L C A S G G A R M	N-P51198
P. yezoensis	Rhodophyta	(150-174)	T R L L E K A T Q E K L P A I I L C A S G G A R M	N-Q1XDT9
C. atmophyticus	Streptophyta	(152-176)	T R L I E H A T N N L L P V I I V C A S G G A R M	N-YP_001019128
C. globosum	Streptophyta	(152-176)	A R L I E Y A T D N S L P L I I V C A S G G A R M	N-NP_683812
S. punctulatum	Streptophyta	(152-176)	T R L I E H A T Q N F L P L I I V C A S G G A R M	N-YP_636397
Z. circumcarinatum	Streptophyta	(152-176)	T R L I E H A T R N R L P L V I V C A S G G A R M	N-YP_636510
ACC (homomeric)				
Yeast (Cytosolic)	Ascomycota	(1605-1629)	N K V T E Y A R K R G I P R I Y L A A N S G A R I	[1]
Rat (Cytosolic)	Chordata	(1698-1722)	L R A S E L A R A E G I P R I Y V A A N S G A R I	[1]
A. thaliana (Plastidial)	Brassicaceae	(1720-1744)	L A V T E L A C T K K L P L I Y L A A N S G A R L	N-NP_174850
A. thaliana (Cytosolic)	Brassicaceae	(1618-1642)	L A V T E L A C A K K L P L I Y L A A N S G A R L	N-NP_174849
T. gondii (Plastidial)	Apicomplexa	(1994-2018)	T Q V S R F S R E Q G L P R V Y I A C N S G A R I	N-AAF04493
T. gondii (GT1)	Apicomplexa	(1645-1669)	T Q V S R F S R E Q G L P R V Y I A C N S G A R I	N-EEE20751
T. gondii (ME49) (Plastidial)	Apicomplexa	(1992-2016)	T Q V S R L S R E Q G L P R V Y I A C N S G A R I	N-XP_002369961
T. gondii (ME49) (Cytosolic)	Apicomplexa	(2726-2750)	Q R A S E I A R K E G I P R I Y I A V N S G A R M	N-XP_002370640
T. gondii (VEG) (Plastidial)	Apicomplexa	(1992-2016)	T Q V S R F S R E Q G L P R V Y I A C N S G A R I	N-EEE30125
T. gondii (VEG) (Cytosolic)	Apicomplexa	(2727-2751)	Q R A S E I A R K E G I P R I Y I A V N S G A R M	N-EEE28725
C. crpytica (Plastidial)	Bacillariophyceae	(1502-1526)	F K A S K Y A R E N K L P R V Y I A C N S G A R I	N-AAA81471
F. cylindrus	Bacillariophyceae	(1671-1695)	K L A S E F A R H M R I P R L Y V A A N S G A R I	J-145710
F. cylindrus	Bacillariophyceae	(1459-1483)	Y A A S K Y A R E N G I P R L Y I S C N A G A R I	J-274583
P. tricornutum	Bacillariophyceae	(1503-1527)	Y K A S V Y A R E R K L P R V Y I A C N A G A R I	J-54926
P. tricornutum	Bacillariophyceae	(1630-1654)	K M A S E Y A R Q R Q I P R L Y V A A N A G A R I	J-55209
T. pseudonana	Bacillariophyceae	(1623-1647)	K M A S V F A R E R R V P R L Y I A A N S G A R I	J-12234
T. pseudonana	Bacillariophyceae	(1501-1525)	F K A S K Y A R E H K L P R V Y I S C N S G A R I	J-6770
B. natans	Chlorarachniophyta	(1425-1449)	F K A S E L A R K E G L P R I F I S A N S G A R I	J-92488
B. natans	Chlorarachniophyta	(1518-1542)	D L A S K R A R A L G L P R F Y F A V N S G A R I	J-18464
Bathycoccus sp.	Chlorophyta	(1517-1541)	F A V S Q Y A R E N N I P R V F L A C N S G A R I	B-Bathy08g01850
Bathycoccus sp.	Chlorophyta	(1621-1645)	R A A M D L A V E L A I P C V Y V S A N S G A R I	B-Bathy11g03860
M. pusilla	Chlorophyta	(1512-1536)	R A A F E L S V T E G L P C V Y I S A N S G A R I	J-16401
M. pusilla	Chlorophyta	(1545-1569)	Y A V S Q Y A R R K G V P R V Y I A S N S G A R I	J-31755
Micromonas sp.	Chlorophyta	(1630-1654)	R A A F D L A V A E G L P C V Y I S S N S G A R I	J-104872
Micromonas sp.	Chlorophyta	(1539-1563)	F A V S Q Y A R Q R G L P R V Y I A S N S G A R I	J-113382
O. lucimarinus	Chlorophyta	(1490-1514)	R A A F D L A V A E G L P C V Y I S S N S G A R I	J-11
O. lucimarinus	Chlorophyta	(1413-1437)	D A V S K Y A R N L G L P R V Y I A S N S G A R I	J-44400
Ostreococcus sp.	Chlorophyta	(1516-1540)	R A A F D L A V E K G L P C V Y I S S N S G A R I	J-36970
Ostreococcus sp.	Chlorophyta	(1414-1438)	D A I S K Y A R N L G L P R V Y I A S N S G A R I	J-61393
V. carteri (Cytosolic)	Chlorophyta	(1942-1966)	R A A V E T S L E E R L P L L Y L A A N A G A R V	J-106840
G. theta	Cryptophyta	(1448-1472)	F K A S E Y A R K R G L P R L Y I A C N S G A R I	J-68771
G. theta	Cryptophyta	(1644-1668)	H L A T S L A Q E E G I P R I F V A A N T G A R I	J-72035
E. huxleyi	Haptophyta	(1435-1459)	E R A S Q F A R E L G V P R I Y M A A N A G A R F	J-449545
E. huxleyi	Haptophyta	(966-990)	D A V S K Y A R A R G L P R L H I A S N S G A R I	J-455280
A. anophagefferens	Pelagophyceae	(1211-1235)	K A A S V Y A R A R G I P R I Y V A C N S G A R I	J-38836
A. anophagefferens	Pelagophyceae	(1581-1605)	Y K V T R Y A A S R G V P R L Y I S S N S G A R I	J-65524
C. merolae (Cytosolic)	Rhodophyta	(1631-1655)	A K A S Q Q A R K L G I P R I Y I A A N S G A R I	CMM188C

Fig. 2.8 Last 25 amino acids of the 51 amino acid carboxylbiotin binding region of β-carboxyl transferase (Roessler and Ohlrogge 1993). Grey highlights the most conservative regions within the heteromeric and homomeric forms. Blue and green species names denote homomeric ACCase positively identified to be found in the cytosol and chloroplast, respectively. Solid lines identify conserved regions in both heteromeric and homomeric ACCase. Details on genera can be found in Supplementary Material 1. (References: [1] Roessler and Ohlrogge (1993), J: Joint Genome Institute, N: NCBI, B: BOGAS, CM: *Cyanidioschyzon merolae* Genome Project).

In contrast to the two CT regions, the ATP binding region of the heteromeric BC subunits and homomeric BC domains show a high degree of sequence conservation (Fig. 2.9). Especially noteworthy is the region with a high accumulation of the amino acid glycine, with a motif of four G interrupted by a single aa before terminating in another G. This motif conforms to the findings of Matte and Delbaere (2001), who state that a high density of glycine is important in the binding of ATP. The intervening aa is completely conserved as R in the heteromeric binding domain and K in the homomeric binding domain (Fig. 2.9). The sixth and seventh aa (KA) are also completely conserved in both types of ACCase (Fig. 2.9).

Another noteworthy point is the motif ENGI at the beginning of the BC sequence of the diatoms (Bacillariophyceae) *Cyclotella cryptica*, *Fragilariopsis cylindrus* and *Thalassiosira pseudonana*, and the motif EEGL in the sequence of *Phaeodactylum tricornutum* (shown in yellow, Fig. 2.9). This could be an distinguishing feature of plastidial ACCase in diatoms, since the second homomeric ACCase sequence of the other three diatoms exhibits the standard GFP motif (Fig. 2.9) and the identity of the presumable plastidial and cytosolic ACCases is consistent with the findings for α-CT as discussed above.

Compared to the homomeric BC domain, the heteromeric BC subunits show an insertion of four additional amino acids, 20 positions after the end of the presented sequences (data not shown). Furthermore, all presented sequences contain the highly conserved carbon dioxide fixation site RDCS (Kimura et al., 2000) 48 and 52 amino acids after the ATP binding regions of the heteromeric and homomeric forms, respectively (data not shown).

There is a clear difference between the homomeric and heteromeric biotin binding site BCCP (data not shown). Most homomeric ACCase contain an MKM motif in their BCCP binding site. The only exceptions are the presumably cytosolic ACCases of the green algal class prasinophyceae (*Ostreococcus* sp., *Ostreococcus lucimarinus*, *Micromonas* sp. and *Micromonas pusilla*), identified through their α-CT binding region sequences, which contain an MKT motif (data not shown). All investigated heteromeric BCCP subunits contain an MKL motif (data not shown). The only exception is the BCCP subunit of *Cyanidioschyzon merolae*, which also contains an MKT motif like the homomeric ACCase of the Prasinophyceae (data not shown). The motif MKL is also found in certain cyanobacteria (Gornicki et al., 1993), and therefore could be unique to cyanobacteria, in contrast to other bacteria like *Escherichia coli*, which contain the standard MKM motif. This difference in the biotin binding region of the different types of

ACCase could be the result of a missense point mutation that does not have an impact on the binding of biotin.

BC (heteromeric)				
E. coli	Bacteria	(153-172)	G Y P V I I K A S G G G G G R G M R V V	[1]
A. variabilis	Cyanobacteria	(153-172)	G Y P V M I K A T A G G G G R G M R L V	N-YP_321035
N. azollae	Cyanobacteria	(153-172)	G Y P V M I K A T A G G G G R G M R L V	N-ZP_03762854
A. thaliana	Brassicaceae	(225-244)	G F P V M I K A T A G G G G R G M R L A	N-O04983
C. reinhardtii	Chlorophyta	(171-290)	G F P V M I K A T A G G G G R G M R L C	J-122970
Chlorella sp.	Chlorophyta	(238-257)	G F P I M I K A T A G G G G R G M R L A	J-140567
V. carteri	Chlorophyta	(194-213)	G Y P I M I K A T A G G G G R G M R L C	J-77540
C. merolae	Rhodophyta	(226-245)	G Y P V M L K A T A G G G G R G I R L V	CMS299C
ACC (homomeric)				
Yeast (Cytosolic)	Ascomycota	(246-265)	G F P V M I K A S E G G G G K G I R Q V	[1]
Rat (Cytosolic)	Chordata	(304-323)	G Y P V M I K A S E G G G G K G I R K V	[1]
A. thaliana (Plastidial)	Brassicaceae	(321-340)	G Y P A M I K A S W G G G G K G I R K V	N-NP_174850
A. thaliana (Cytosolic)	Brassicaceae	(219-238)	G Y P A M I K A S W G G G G K G I R K V	N-NP_174849
T. gondii (Plastidial)	Apicomplexa	(521-540)	G Y P M M I K A S E G G G G K G I R M V	N-AAF04493
T. gondii (Cytosolic)	Apicomplexa	(409-428)	G Y P V M I K A S E G G G G K G I R R V	N-AAF04494
T. gondii (GT1)	Apicomplexa	(172-191)	G Y P M M I K A S E G G G G K G I R M V	N-EEE20751
T. gondii (ME49) (Plastidial)	Apicomplexa	(521-540)	G Y P M M I K A S E G G G G K G I R M V	N-XP_002369961
T. gondii (ME49) (Cytosolic)	Apicomplexa	(384-403)	G Y P V M I K A S E G G G G K G I R R V	N-XP_002370640
T. gondii (VEG) (Plastidial)	Apicomplexa	(521-540)	G Y P M M I K A S E G G G G K G I R M V	N-EEE30125
T. gondii (VEG) (Cytosolic)	Apicomplexa	(385-404)	G Y P V M I K A S E G G G G K G I R R V	N-EEE28725
C. crpytica (Plastidial)	Bacillariophyceae	(287-307)	E N G I M I K A S E G G G G K G I R F V	N-AAA81471
F. cylindrus	Bacillariophyceae	(272-291)	E N G I M V K A S E G G G G K G I R F V	J-274583
F. cylindrus	Bacillariophyceae	(186-205)	G F P I M I K A S E G G G G K G I R M C	J-145710
P. tricornutum	Bacillariophyceae	(280-299)	E E G L M I K A S E G G G G K G I R F V	J-54926
P. tricornutum	Bacillariophyceae	(221-240)	G F P V M I K A S E G G G G K G I R K V	J-55209
T. pseudonana	Bacillariophyceae	(286-305)	E N G I M I K A S E G G G G K G I R F V	J-6770
T. pseudonana	Bacillariophyceae	(186-205)	G F P I M I K A S E G G G G K G I R M V	J-12234
B. natans	Chlorarachniophyta	(244-263)	G F P V M L K A S E G G G G K G I R M S	J-92488
B. natans	Chlorarachniophyta	(205-224)	G F P V M I K A S E G G G G K G I R M C	J-18464
Bathycoccus sp.	Chlorophyta	(281-300)	G F P V M L K A S E G G G G K G I R M S	B-Bathy08g01850
Bathycoccus sp.	Chlorophyta	(203-222)	G Y P V M I K A S E G G G G K G I R M V	B-Bathy11g03860
Chlorella sp. (Cytosolic)	Chlorophyta	(235-254)	G Y P V M L K A S W G G G G K G I R K V	J-56737
M. pusilla	Chlorophyta	(190-209)	G F P L M V K A S E G G G G K G I R M V	J-16401
M. pusilla	Chlorophyta	(291-210)	G Y P V M L K A S E G G G G K G I R M S	J-31755
Micromonas sp.	Chlorophyta	(192-211)	G F P L M I K A S E G G G G K G I R M V	J-104872
Micromonas sp.	Chlorophyta	(286-305)	G Y P V M L K A S E G G G G K G I R M S	J-113382
O. lucimarinus	Chlorophyta	(189-208)	G F P V M I K A S E G G G G K G I R K V	J-11
O. lucimarinus	Chlorophyta	(172-191)	G F P V M L K A S E G G G G K G I R M S	J-44400
Ostreococcus sp.	Chlorophyta	(211-230)	G F P V M V K A S E G G G G K G I R K V	J-36970
Ostreococcus sp.	Chlorophyta	(172-191)	G F P V M L K A S E G G G G K G I R M S	J-61393
V. carteri (Cytosolic)	Chlorophyta	(288-307)	G Y P V M L K A S W G G G G K G I R K V	J-106840
G. theta	Cryptophyta	(219-238)	G Y P V M L K A S E G G G G K G I R M S	J-68771
G. theta	Cryptophyta	(224-243)	G Y P L M V K A S E G G G G K G I R K V	J-72035
E. huxleyi	Haptophyta	(206-225)	G Y P I M I K A S E G G G G K G V R M A	J-449545
A. anophagefferens	Pelagophyceae	(229-248)	G Y P V M L K A S E G G G G K G I R M S	J-38836
A. anophagefferens	Pelagophyceae	(239-258)	G Y P V M L K A S E G G G G K G I R M N	J-65524
C. merolae (Cytosolic)	Rhodophyta	(213-232)	G Y P V M I K A S E G G G G K G I R L C	CMM188C

Fig. 2.9 ATP binding region biotin carboxylase (Roessler and Ohlrogge 1993). Grey highlights the most conservative regions within the heteromeric and homomeric forms. Blue and green species names denote homomeric ACCase positively identified to be found in the cytosol and chloroplast, respectively. Yellow highlights a deviation of the general pattern occurring in diatoms. Solid lines identify conserved regions in both heteromeric and homomeric ACCase. Details on genera can be found in Supplementary Material 1. (References: [1] Roessler and Ohlrogge (1993), J: Joint Genome Institute, N: NCBI, B: BOGAS, CM: *Cyanidioschyzon merolae* Genome Project).

5. Impact of enzymes involved in lipid synthesis on lipid yield in algae

Here we briefly discuss the biochemical properties of acetyl-CoA carboxylase (ACCase) and the impact of different enzymes that are part of the lipid synthesis pathway on lipid yield.

As a key enzyme, ACCase is highly regulated on a genetic and biochemical level (Sasaki and Nagano, 2004). This can also be seen in the levels of the substrate acetyl-CoA being higher compared to the theoretical thermodynamic equilibrium relative to the measured concentrations, which is typical for regulated enzymes (Ohlrogge and Jaworski, 1997). It has been shown, that the supply of acetyl-CoA is not rate limiting since levels of acetyl-CoA do not change considerably under different levels of fatty acid (FA) synthesis (Ohlrogge and Jaworski, 1997).

In plants with heteromeric plastidial ACCase, light-induced changes in pH und Mg^{2+} concentration in the plastid stroma activate the ACCase (Sasaki and Nagano, 2004). Light induces a change from approximately pH 7 to 8 (Werdan et al., 1975) and an increase in Mg^{2+} concentration in the stroma (Portis and Heldt, 1976). The pH optimum for ACCase activity lies between 7.5 and 8.5 (Charles and Cherry, 1986, Egin-Buhler and Ebel, 1983, Mohan and Kekwick, 1980, Nikolau and Hawke, 1984, Slabas and Hellyer, 1985). Furthermore, Mg^{2+} is essential for the function of ACCase due to the requirement of Mg^{2+}-ATP for the reaction (Finlayson and Dennis, 1983, Mohan and Kekwick, 1980, Nielsen et al., 1979, Sauer and Heise, 1984). Therefore, conditions in chloroplasts exposed to light provide optimal conditions for maximal ACCase activity. Additionally, the synthesis of FAs requires a large amount of energy (in the form of Mg^{2+}-ATP) and reductive potential (in the form of NADPH), which are the products of photosynthesis. ACCase has been purified and characterised in several plants, including avocado (Mohan and Kekwick, 1980), maize (Nikolau and Hawke, 1984), parsley (Egin-Buhler and Ebel, 1983), spinach (Kannangara and Stumpf, 1972), and wheat (Egin-Buhler et al., 1980), but only in two microalgae: *Cyclotella cryptica* (Bacillariophyceae) (Roessler, 1990) and *Isochrysis galbana* (Haptophyta) (Livne and Sukenik, 1990).

Several studies have been conducted on genetically modified organisms in which ACCase has been overexpressed. Genetically modified rape (*Brassica napus*) and potato plants (*Solanum tuberosum),* showed that an overexpression of homomeric ACCase increased ACCase activity, which resulted in a significantly higher lipid content in rape seeds (Roesler et al., 1997) and potato tubers (Klaus et al., 2004). In contrast, a similar overexpression of the nuclear encoded *acc1* gene (cloned from *Cyclotella*

cryptica), which is targeted to the plastid, resulted in an increase of ACCase gene expression and activity in *C. cryptica* and *Navicula saprophila*, but no significant increase in lipid content was observed (Dunahay et al., 1996, Dunahay et al., 1995, Sheehan et al., 1998). This result could be explained by a feedback inhibition mechanism countering the increased ACCase activity downstream of ACCase (Sheehan et al., 1998). However, the targeting of the enzyme to the chloroplast has not been confirmed and therefore increased enzyme activity could be located in the cytosol. So far, only one study investigated the gene expression of ACCase in algae. The expression of plastidial heteromeric ß-CT and cytosolic homomeric ACCase has been investigated in *Chlorella sorokiniana (Wan et al., 2011)*. The study showed that while ß-CT was upregulated during stationary phase, along with an increase in lipid content, the expression of cytosolic ACCase was not increased. This confirms that cytosolic ACCase is not involved in *de novo* FA synthesis.

It has been shown in yeast (*Saccharomyces cerevisiae*) that the gene encoding for ACCase and the two unlinked genes (FAS1 and FAS2), which make up the fatty acid synthase (FAS) complex, are regulated by coordinated expression (Chirala, 1992, Schüller et al., 1992). A similar coordinated expression of the different subunits of type 2 FAS seems to occur in plants (Ohlrogge and Jaworski, 1997). However, expression levels appear to be below the theoretical maximum, leaving room for higher expression (Ohlrogge and Jaworski, 1997). Unfortunately, the multipart nature of type 2 FAS complicates genetic engineering, as overexpression of individual subunits results in non-viable organisms, reduced growth or reduced lipid synthesis (Courchesne et al., 2009).

Manipulation of the different acyl-ACP thioesterases (AATs) present in the chloroplast changes the length of the FA produced by FAS (reviewed in Thelen and Ohlrogge (2002)). Theoretically, this does not change the total amount of FA produced, just the length. However, it was shown that the production of FAs with unnatural length in plants triggers degradation of these FAs rather than incorporation into lipids (Thelen and Ohlrogge, 2002). Interestingly, overexpression of AATs did not significantly reduce lipid productivity, suggesting up-regulation of ACCase and FAS to compensate for the loss of foreign FA to β–oxidation (Thelen and Ohlrogge, 2002).

Overexpression of lysophosphatidate acyl-transferase (LPAAT) in rape resulted in an overall increase in triacylglycerides (TAGs) in oilseeds, along with an increase in long-chained FAs in the TAGs (Zou et al., 1997). The overexpression of acyl-CoA:diacylglycerol acyl-transferase (DGAT), also resulted in an increase in TAG

accumulation in yeast (Bouvier-Navé et al., 2000), tobacco (Bouvier-Navé et al., 2000) and *Arabidopsis* (Jako et al., 2001). This increase in TAG content suggests that overexpressing LPAAT or DGAT directs the usage of phosphatidate and diacylglycerol (DAG), which are precursors for the synthesis of phospholipids and TAGs (Fig. 2.1), for TAG rather than phospholipid production (Courchesne et al., 2009). Unfortunately, potential changes in phospholipid content were not quantified, neither was ACCase activity, which hypothetically should have been upregulated to provide the FAs required, unless a large FA pool exists.

The outcomes of overexpression studies conducted to date suggest that overexpression of enzymes involved in the endoplasmic reticulum localised part of the pathway draws on a transient FA pool. Diminishing FA pools would then exert a positive feedback control for ACCase and potentially stimulating FA synthesis (Courchesne et al., 2009). Whether overexpression of LPAAT and DGAT results in maximal activity of ACCase and FAS, which under normal circumstances do not operate at maximum capacity, or whether it leads to increased expression of the co-regulated ACCase and FAS genes remains to be investigated. In summary, overexpression of ACCase alone would not likely result in increased TAG content, unless the draw on the transient FA pool, by other processes located further down the pathway, is increased simultaneously.

6. Conclusions

We have shown that the general belief that most algae contain heteromeric ACCase in their plastids is incorrect. Only red and green algal plastids derived from a primary endosymbiotic event, except for the green algal class Prasinophyceae, contain heteromeric plastidial ACCase. In contrast, investigated phyla with plastids derived from a secondary endosymbiotic event with a red alga (Cryptophyta, Stramenopiles, Haptophyta and Apicomplexa) and a green alga (Chlorarachniophyta) contain homomeric plastidial ACCase. In addition, the absence of the *accD* gene in the plastid genome of the non-photosynthetic euglenoid flagellate *Astasia longa* (Gockel and Hachtel, 2000) indicates that the second phylum of the green lineage of secondary endosymbiosis follows a same pattern. It will be interesting to see whether the current phylogenetic plastidial ACCase pattern will be confirmed, once sequences of species belonging to the missing taxa (namely the Glaucocystophyta and Euglenophyta) become available and more species of the taxa investigated in this chapter are sequenced.

The distinctive difference in the substrate binding regions of heteromeric and homomeric α-CT and β-CT will be useful for a preliminary differentiation between the two ACCase types in sequences derived from Next Generation Sequencing. Furthermore, the acetyl-CoA binding region of homomeric α-CT can be used to distinguish between cytosolic and plastidial ACCase.

Previous research showed that that overexpression of homomeric ACCase does not necessarily increase lipid content in algae, but it is unclear whether it was indeed targeted to the plastid, the only site of *de novo* FA synthesis. In addition, the existence of negative feedback regulation via the FA pool, as well as enzymatic bottlenecks further down the lipid synthesis pathway could have prevented a detectable increase in TAG content, which requires further investigation. Overexpression studies carried out in algae appear to suggest that overexpression of ACCase should at least be accompanied by overexpression of enzymes operating in the ER localised part of the lipid synthesis pathway in order to simultaneously increase the removal of FAs from the transient FA pool. Additionally, impacts of environmental and physicochemical parameters on enzyme regulation need to be unravelled.

It is also unknown if the type of ACCase present in the chloroplast affects lipid productivity. Other factors including growth rate, culture age, nutrient availability, temperature and light all affect lipid content and therefore lipid productivity. It therefore is difficult to isolate the effect of ACCase type on lipid content and further research in this direction is required.

Chapter 3 - Resolving conflicting phylogenetic views on algal taxonomy using cytosolic and plastidial acetyl-CoA carboxylase

The following chapter is a collaborative effort of which each author's contribution is outlined below.

Contribution

Roger Huerlimann: Conception and execution of project, writing and editing chapter
Kyall R. Zenger: Supervision, editing
Dean R. Jerry: Supervision, editing
Kirsten Heimann: Supervision, editing

This chapter is currently in preparation as a publication in *PLOS ONE* with the following authors: Roger Huerlimann, Kyall R. Zenger, Dean R. Jerry and Kirsten Heimann

1. Abstract

Algal evolution is still hotly debated and our understanding is being impeded by an unknown number of endosymbiotic events that gave rise to plastids and the acquisition of genes through horizontal gene transfer (HGT). The Alveolata (Dinoflagellata and Apicomplexa) and Chromista (Stramenopiles, Haptophyta and Cryptophyta) have traditionally been grouped together as Chromalveolata, forming the red lineage. However, recent genetic evidence supports grouping the Stramenopiles, Alveolata and the green plastid containing Rhizaria as SAR, to the exclusion of Haptophyta and Cryptophyta. Multiple endosymbiotic events are suggested to explain the observed genetic diversity. However, additional genetic evidence is required to resolve these conflicting views. The unique properties of acetyl-CoA carboxylase (ACCase) provide a potential solution. In algae, ACCase exists in two locations (cytosol and plastid) and in two forms (homomeric and heteromeric). ACCase can therefore inform on the phylogenetic relationships of hosts and their plastids, allowing different theories of endosymbiotic events to be tested. Plastidial and cytosolic homomeric ACCase sequences were isolated from *I.* aff. *galbana* (TISO), *Chromera velia* and *Nannochloropsis oculata,* representing three taxonomic groups for which ACCase sequences were lacking. Phylogenetic analyses show that cytosolic ACCase strongly supports the SAR to the exclusion of all other taxa. Conversely, plastidial ACCase groups the SAR with the Haptophyta, Cryptophyta and Prasinophyceae (Chlorophyta). These two ACCase based, phylogenetic relationships suggest that the plastidial homomeric ACCase was acquired by the Haptophyta, Cryptophyta and SAR, before the photosynthetic Rhizaria acquired their green plastid. Additionally, plastidial ACCase was not derived from a duplication of the cytosolic ACCase, but by HGT from an ancestor or relative of Prasinophyceae.

2. Introduction

Entering into an endosymbiotic relationship, in the form of an internal mutualist, offers the evolutionary advantage of gaining access to new biochemical pathways, resulting in increased competitiveness (Wernegreen, 2012). These endosymbiotic relationships provide many advantages and have shaped the world we know to a high degree. For example, the endosymbiosis between cnidarians and dinoflagellates has allowed corals to use the photosynthetic capacity of the endosymbiont to obtain additional energy. Of even greater importance were the more permanent endosymbiotic events which led to the evolution of mitochondria and chloroplasts (plastids) as organelles, enabling eukaryotes to prosper in an oxygen enriched atmosphere and to assimilate inorganic carbon through oxygenic photosynthesis (Wernegreen, 2012).

Eukaryotic life is currently divided into six major supergroups, comprising Opisthokonta, Amoebozoa, Archaeplastida, Rhizaria, Chromalveolata and Excavata (Adl et al., 2005, Keeling et al., 2005), of which the last four contain photosynthetic members. Endosymbiotic events were a driving factor in the evolution and diversification of photosynthetic organisms, especially algae (Bhattacharya et al., 2004). From a parsimonious point of view, endosymbiotic events are considered to be rare due to their complexity (Archibald, 2012). However, the observed algal diversity is difficult to explain in the most parsimonious way, and plastid diversity points to at least five endosymbiotic events, not including possible multiple endosymbiotic events in Dinoflagellata.

2.1 Traditional view on algal phylogeny

The Archaeplastida include the Viridiplantae (land plants and green algae), the Rhodophyta (red algae) and the Glaucocystophyta (a small group of freshwater microalgae) (Ball et al., 2011), all containing plastids surrounded by two envelope membranes. There is strong evidence that the plastids of these three groups evolved from a single primary endosymbiotic event involving a cyanobacterium (Archibald, 2012). Even though the primary endosymbiosis of a cyanobacterium is considered to only have occurred once, there is an euglyphid testate amoeba which has recently (in an evolutionary sense) taken up a cyanobacterium in what seems to be an independent primary endosymbiotic event (reviewed in Keeling (2010)). The majority of algal diversity, however, appears to be a testament of secondary and tertiary endosymbiotic events.

In secondary endosymbiosis, a heterotrophic eukaryote took up a photosynthetic eukaryote containing a plastid derived from primary endosymbiosis. At least three secondary endosymbiotic events gave rise to a large number of highly diverse organisms with plastids derived either from a red or green alga. The red lineage, first proposed by Cavalier-Smith (1998, 1999), is composed of the Chromalveolata, joining the former supergroups Chromista (containing the Cryptophyta, Haptophyta and Stramenopiles) and Alveolata (comprising Apicomplexa, Chromerida, Ciliophora and Dinoflagellata). The green lineage is comprised of the Chlorarachniophyta (Rhizaria) and Euglophyta (Excavata), which have taken up their plastids in two independent endosymbiotic events from an ancestral core Chlorophyta (Ulvophyceae-Trebuxiophyceae-Chlorophyceae) and a Prasinophyceae, respectively (Rogers et al., 2007, Turmel et al., 2009). This is supported by the former host organisms of Rhizaria and Excavata not being closely related, while traditionally a close phylogenetic relationship between all Chromalveolata has been reported (Keeling, 2010, Archibald, 2009a).

2.2 Advances in algal taxonomy

Recently, the support for the Chromalveolata grouping has been waning (Archibald, 2012). While there is strong evidence that all Chromalveolates contain a red plastid from a secondary endosymbiotic event with a red alga, it is still controversial how many endosymbiotic events occurred (Keeling, 2010, Bodył et al., 2009). Newer phylogenetic analyses group the Stramenopiles and Alveolata together with the Rhizaria (abbreviated as SAR), with Haptophyta as a sister group and the Cryptophyta being more closely related to the Viridiplantae (Burki et al., 2012). This provides additional evidence against the monophyly of the Chromalveolata.

To further complicate matters, genes that are phylogenetically related to green algae have been found in diatoms (Stramenopile) (Moustafa et al., 2009), *Chromera velia* (Chromerida) (Woehle et al., 2011) and numerous species of Stramenopile, Haptophyta and Cryptophyta (Frommolt et al., 2008, Petersen et al., 2006). However, a hypothesis that the red lineage contained an ancestral cryptic plastid of green origin has largely been refuted (Archibald, 2009a, Deschamps and Moreira, 2012). The presence of green algal genes in the Chromalveolata is mostly explained by either sample bias (Deschamps and Moreira, 2012, Dagan and Martin, 2009) or horizontal gene transfer (Archibald, 2012) and their presence cannot be taken as proof for endosymbiotic gene transfer. Nevertheless, the single origin of Chromalveolata cannot explain all the findings of recent phylogenetic studies and less parsimonious solutions,

including secondary and tertiary endosymbiosis, are invoked to explain the inconsistencies. For example, a single secondary endosymbiotic event was proposed, where an ancestral Cryptophyte took up a red alga, followed by a tertiary event that led to the Haptophytes, Stramenopiles and Alveolata (Archibald 2012). This hypothesis also offers an explanation for the occurrence of heterotrophic members in the Chromalveolata, which share a common ancestor with the Haptophytes, Stramenopiles and Alveolata, but do not contain a plastid, avoiding assumptions of multiple plastid losses (Bodył et al., 2009). Overall, algal phylogeny is a complicated topic and many questions still go unanswered, awaiting more genetic data to become available.

Acetyl-CoA carboxylase (ACCase) is involved in the highly conserved fatty acid synthesis pathway. Two forms of ACCase exist, the multi-subunit, prokaryotic, heteromeric form and the multi-domain, eukaryotic, homomeric form. While all eukaryotes contain the homomeric form of ACCase in their cytosol, the type of the plastidial form can differ between taxa (see Huerlimann and Heimann (2013) for detail). All Archaeaplastida contain heteromeric ACCase in their plastids, with the exception of the green algal group Prasinophyceae and certain plants (mainly the true grasses Poaceae). All investigated organisms with a plastid derived through secondary or tertiary endosymbiosis, conversely, contain homomeric ACCase in their plastids (Huerlimann and Heimann, 2013).

While single gene analyses generally lack potential to provide irrefutable evidence for the evolution of plastids (Archibald 2012), the use of an enzyme that occurs in two different cellular compartments: the plastid (including the apicoplast, a plastid that no longer carries out photosynthetic function, but is essential for fatty acid, isoprenoid and haeme synthesis (McFadden and Waller, 1997, Waller and McFadden, 2005), of the Apicomplexa) and the cytosol, could be useful for dissecting evolutionary relationships. The homomeric form of ACCase is always encoded in the nucleus and can be targeted at the cytosol and the plastids of some photosynthetic organisms. In contrast, only the genes for three subunits of the heteromeric form of ACCase, if present, are encoded in the nucleus, while the gene for the fourth subunit, β-carboxyltransferase, is encoded in the plastidial genome. The heteromeric plastidial form of ACCase is derived from the original cyanobacterial endosymbiont, while the homomeric plastidial form can be either derived from the cytosolic ACCase of the endosymbiont, the host or horizontal gene transfer. However, the complexity of identifying whether horizontal and endosymbiotic gene transfers have occurred makes it difficult to resolve the question of algal taxonomy (Archibald 2012). The dual nature of ACCase (cytosolic and plastidial) and the possibility of various origins of the plastidial form may provide a way to further

unravel the unresolved phylogeny of algae. Therefore, ACCase could serve as a powerful tool to show the relationship between hosts (cytosolic ACCase) and help distinguishing between the various possibilities of the origin of the genes whose products are targeted at the plastid (plastidial heteromeric ACCase for primary endosymbiosis and plastidial homomeric ACCase for secondary/tertiary endosymbiosis).

In the present study, ACCase sequences were isolated from *Isochrysis.* aff. *galbana* (TISO; Haptophyta), *Chromera velia* (Chromerida) and *Nannochloropsis oculata* (Eustigmatophyceae), representing three taxonomic groups for which ACCase sequences were not yet well represented. These new sequences were incorporated with publicly available ACCase sequences to exhaustively examine the phylogeny of the plastidial and cytosolic form of homomeric ACCase and to integrate these findings with our current taxonomic understanding of algae. Phylogenetic analyses based on cytosolic ACCase were used to add information to the relationship of the hosts, while information of the plastidial ACCase aids to further unravel the origin of genes targeted at plastids derived from secondary/tertiary endosymbiosis. Furthermore, we investigate the relationship of the plastidial encoded β-carboxyltransferase subunit in Archaeaplastida and Cyanobacteria.

3. Material and Methods

3.1 Algal culture

Isochrysis sp. (CS-177) was obtained from CSIRO, *Chromera velia* was isolated from Sydney Harbour, New South Wales, Australia (Moore et al., 2008) and *Nannochloropsis oculata* was isolated from the Great Barrier Reef. All species were kept in the culture collection of the North Queensland Algal Identification / Culturing Facility (NQAIF) at James Cook University (JCU) in Townsville, Australia.

Algae were cultured in unpolluted ocean seawater (Orpheus Island, Great Barrier Reef, Queensland, Australia), that was filtered through a membrane (pore size 0.2 µm), autoclaved and enriched with L1 medium (Andersen et al., 2005). Cultures were maintained in 1 L L1-medium in 2 L Erlenmeyer flasks at 24 °C, an L:D cycle of 12:12 h, and a photon flux intensity of 138 µmol m^{-2} s^{-1} provided by cool white fluorescent lights. For harvest, an aliquot was taken and cell density determined. The cells were then centrifuged at 3,000 xg for 20 min and frozen in liquid nitrogen for use in total RNA extraction.

3.2 Total RNA extraction

Microalgal cells were homogenised with a liquid nitrogen cooled mortar and pestle. Total RNA was extracted from the homogenised cells with Ultra Spec RNA Isolation Reagent (Fisher Biotec, Wembley, Australia). Samples were DNase treated with the Turbo DNA-free™ kit (Applied Biosystems, Life Technologies, Carlsbad, CA), followed by a NH_4 precipitation. RNA quality and DNA contamination were confirmed by 1% agarose gel electrophoresis with post-gel staining. RNA concentration and purity were checked on a NanoDrop spectrophotometer using 230/260 and 260/280 nm wavelengths, as well as on an Agilent bioanalyser.

3.3 Illumina sequencing

The library construction, duplex-specific thermostable nuclease (DSN) normalisation, sequencing and de-multiplexing were performed by the BioMedical Genomics Center of the University of Minnesota. The samples were multiplexed on one lane, together with two species not presented in this publication. The TruSeq RNA libraries were created with 100 ng of poly-A selected/enriched total RNA. The samples were added to the TruSeq RNA workflow at step 11 of "Make RFP" and were processed as written in the manual.

The DSN treatment was carried out as per manual. The resulting libraries underwent quality control by Agilent HS, PicoGreen, and Kapa qPCR. Of each library, 250 ng was pooled and cut using a Pippin Prep (Sage Science) for a target size of 320 +/- 5%. The final library pool was run on an Agilent HS at 1:10 and quantified by PicoGreen before flow cell clustering. Size selected and pooled libraries for all samples were clustered at 10pM on a v1.5 Illumina flow cell. Libraries were sequenced on a paired end 100bp multiplexed sequencing run. Primary analysis and de-multiplexing of the flow cell were performed using CASAVA 1.7.

3.4 *De novo* transcriptome assembly

A preliminary assembly, created in Velvet (version 1.1.07; https://github.com/dzerbino/velvet; Zerbino and Birney (2008)) and Oases (version 0.1.23; https://github.com/dzerbino/oases; Schulz et al. (2012)), was used to determine insert sizes in Bowtie (version 0.12.7; http://bowtie-bio.sf.net; Langmead et al. (2009)). The statistical program R (version 2.13.1; http://www.r-project.org/) was then used to find the mean and standard deviation values for the insert sizes created in Bowtie.

Isochrysis aff. *galbana* (TISO) was assembled in Velvet (version 1.1.07) and Oases (version 0.1.23), using k-mer lengths of 51, 53, 55 and 57, the results of which were then concatenated. *Chromera velia* and *Nannochloropsis oculata* were assembled in Velvet (version 1.1.07) and Oases (version 0.2.01), using the merge pipeline with a k-mer range of 51, 53, 55, 57 and 59. The pipeline automatically concatenated the outputs of the different k-mer lengths, but there was no noticeable reduction in number of transcripts, making a collapsing step necessary.

The program CD-HIT-EST (http://www.bioinformatics.org/cd-hit/; Li and Godzik (2006)) was used to remove redundant transcripts in the concatenated transcriptomes, with a sequence identity threshold of 0.99, a word size of 10, the “accurate but slow” mode of the algorithm and comparing both strands and the length of throw_away_sequences set to 20.

3.5 Functional annotation

Automated putative annotations for candidate genes were performed in Blast2GO (version 2.5.0; *www.blast2go.org;* Conesa et al. (2005)) based on sequence similarity searches (BLASTX) against the NCBI Blast database (E-value ≤ 10^{-3}). Annotated transcripts were then screened for genes of interest and imported into Geneious Pro (version 5.6.6; www.geneious.com; Drummond et al. (2011)) for further manual annotation and assembly. Final nucleotide (nt) assemblies were translated into amino acid (aa) sequences in Geneious Pro and visually checked for the correct reading frame with the help of sequences from related species obtained from BLASTX results. Identified ACCase nt and aa sequences were deposited on NCBI under the following access numbers: KF673905 to KF673101.

3.6 Phylogenetic analysis

Phylogenetic analysis was performed on amino acid sequences. Sequences not produced in this study were obtained from NCBI (www.ncbi.nlm.nih.gov) and JGI (genome.jgi-psf.org). Several partial ACCase sequences of short length were excluded from the alignment (notably plastidial ACCase from *Emiliania huxleyi* and *Symbiodinium* Clade C and all sequences from *Ectocarpus siliculosus*). The β-carboxyltransferase dataset includes all known algal sequences of β-CT by March 2013, together with selected sequences of cyanobacteria and land plants.

Sequences were aligned with MUSCLE (version 3.6; Edgar (2004)) in Geneious Pro. GBlocks (version 0.91b; molevol.cmima.csic.es/castresana/Gblocks.html; Castresana (2000) and Talavera and Castresana (2007)) was used to eliminate divergent regions

and poorly aligned positions using standard settings. The resulting dimensions (number of taxa x number of amino acid positions) for the two alignments were as follows: β-CT, 38 x 213 and ACCase 55 x 564. The amino acid alignments are available request. Models were tested and chosen with ProtTest (version 3.2; darwin.uvigo.es/software/prottest3; Darriba et al. (2011)) to find the best fitting model with AIC, using a maximum-likelihood (ML) starting tree. The most suitable models were CpREV +G and LG+G+F for the β-CT and ACCase datasets, respectively. Bayesian inference (BI) and Maximum Likelihood (ML) analyses were performed on both datasets. BI trees were used for the publication.

ML trees were performed in PhyML (version 3.0; www.atgc-montpellier.fr/phyml; Guindon and Gascuel (2003)), using the models mentioned above with 4 rate categories, gamma estimated from the data and 2000 bootstraps. Bayesian trees were performed in MrBayes (version 3.2; available from mrbayes.sourceforge.net; Ronquist and Huelsenbeck (2003)) for 2 x 10^6 generations (ACCase) and 3 x 10^6 generations (β-CT), running 4 chains in parallel (3 heated) with a sampling frequency of 2,500 and a diagnostic frequency of 25,000. A four-category gamma model was used, as suggested by ProtTest, with the alpha parameter being estimated from the data during the run. The aa models were set to “mixed” to let Mr. Bayes determine the most suitable model, which was Cprev+G for β-CT and WAG+G for ACCase. The average standard deviation of split frequencies was used to evaluate the convergence of the sampled chains and a 25% burn-in fraction was chosen for each analysis.

FigTree (version 1.3.1; tree.bio.ed.ac.uk/software/figtree) was used to display finished trees. The β-CT trees were rooted using *Escherichia coli*, while the ACCase trees were midpoint rooted.

4. Results and Discussion

4.1 Homomeric acetyl-CoA carboxylase characterisation

We identified five previously unsequenced homomeric ACCase transcripts from *Nannochloropsis oculata* (two), *I.* aff. *galbana* (TISO) (one) and *Chromera velia* (two).The identification through BLASTX searches of the transcripts showed close relationships with other existing homomeric ACCase transcripts (Table 3.1). The length ranged from 2,082 to 2,362 amino acids and is comparable to that of other transcripts of algal ACCase. One transcript of *N. oculata* is complete with start and stop codon, while the other transcripts either contain a stop codon only (*N. oculata* and *I.* aff. *galbana* (TISO)), or are missing the start and stop codon (*C. velia*). One of the

transcripts for *C. velia* could not be assembled completely and consisted of three fragments (Table 3.2).

Table 3.1 BLASTX results of putative ACCase nt sequences of *N. oculata* (2 sequences), *I.* aff. *galbana* (TISO) (1 sequence) and *C. velia* (2 sequences)

Species	NCBI accession #	Closest identifiable match on Genbank (% identity)	E-value	NCBI accession # of closest match	Defined function
N. oculata	KF673100	ACCase, *Thalassiosira pseudonana* (57.5)	0	XP_002296083	ACCase
	KF673101	ACCase, (putative) *Phytophthora infestans* (46.9)	0	XP_002997355	ACCase
I. aff. *galbana* (TISO)	KF673099	ACCase, *Rhizopus delemar* (40.6)	0	EIE80272	ACCase
C. velia	KF673098	ACCase, *Thalassiosira pseudonana* (51.9)	0	XP_002296083	ACCase
	KF673096	ACCase, *Toxoplasma gondii* (51.2)	0	EPR63797	ACCase
	KF673097	ACC2, *Toxoplasma gondii* (58.1)	2.6E-102	AAK16498	ACCase
	KF673095	ACCase, *Toxoplasma gondii* (63.3)	0	EPR63797	ACCase

Table 3.2 Final transcripts after assembly and identification. Plastidial ACCase (ACC1) and cytoplasmic ACCase (ACC2). Abbreviations of regions: Start codon (start), ATP binding (BC), biotin binding (BCCP), carboxyl binding (β-CT), acetyl-CoA binding (α-CT), stop codon (stop).

Species	NCBI accession #	Gene	Localisation	nt Length	aa Length	Regions included
N. occulata	KF673100	ACC1	Plastid	6,414	2,317	start, BC, BCCP, β-CT, α-CT, stop
	KF673101	ACC2	Cytosol	7,089	2,362	BC, BCCP, β-CT, α-CT, stop
I. aff. *galbana* (TISO)	KF673099	ACC1	Plastid	6,402	2,133	BC, BCCP, β-CT, α-CT, stop
C. velia	KF673098	ACC1	Plastid	6,246	2,082	BC, BCCP, β-CT, α-CT
	KF673096	ACC2	Cytosol	3,345	1,115	BC, BCCP
	KF673097	ACC2	Cytosol	858	286	β-CT
	KF673095	ACC2	Cytosol	1,260	420	α-CT

Further manual identification was undertaken by using the four binding sites, biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), β- and α-carboxyltransferase (β-CT and α-CT), (described in detail in Huerlimann and Heimann (2013)), which were found in all transcripts (Table 3.2). The first three amino acids of the acetyl-CoA binding site of the α-CT region were used to identify the cytosolic or plastidial localisation of the transcripts (see Huerlimann and Heimann (2013)). One transcript each of *C. velia* and *N. oculata* contained an AKS motif and were therefore identified as cytosolic ACCase (*ACC2*) (Fig. 3.1). Another two transcripts from *I.* aff. *galbana* (TISO) and *N. oculata* exhibited the motif GKS, indicating their plastidial localisation (*ACC1*). The α-CT region of *ACC1* from *C. velia* contained the amino acid motif GKG, which deviates from the usually very conserved plastidial motif (Fig. 3.1). The transcript was still putatively identified as being targeted to the plastid, based on the cytosolic nature of the first transcript of *C. velia*.

Fig. 3.1 Acetyl-CoA binding site of the α-CT region. First three amino acids were used to distinguish between cytosolic (AKS or AKT) and plastidial (GKS or GKG) ACCase.

4.2 Homomeric acetyl-CoA carboxylase phylogenetics

The Chromalveolate theory is still hotly debated (Baurain et al., 2010, Archibald, 2012). The traditional view fails to explain recent genetic evidence (e.g. the relationship of the SAR species to the exclusion of the Haptophyta and Cryptophyta), and the latest phylogenetic explanations invoke additional endosymbiotic events to explain inconsistencies (Archibald 2012). In order to unravel the phylogenetic relationship of acetyl-CoA carboxylase (ACCase) between the different taxa, phylogenetic consensus trees for plastidial and cytosolic ACCase were constructed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods (Fig. 3.2). This dataset included all algal sequences of ACCase found on Genbank and JGI (accessed March 2013), to the exclusion of all the sequences of *Ectocarpus siliculosus*, the plastidial sequence of *Emiliania huxleyi* and the cytosolic sequence of one *Toxoplasma gondii* strain, which were incomplete and missed important binding regions. The sequence for the cytosolic ACCase of *Chromera velia* was also in three fragments, but included all four important binding regions (Table 3.2). Of the five major nodes (A to E), nodes B to E were strongly supported (BI = 100%, ML ≥ 99%), while node A was strongly supported by BI (BI = 100%, ML > 50%) (Fig. 3.2). Node A, B, C and D contain cytosolic ACCase only, with the exception of the plastidial ACCase from plants. Conversely, node E, contains mainly plastidial ACCase. Therefore, nodes A through D show the relationship of the hosts, while node E provides information on the origin of the plastidial ACCase.

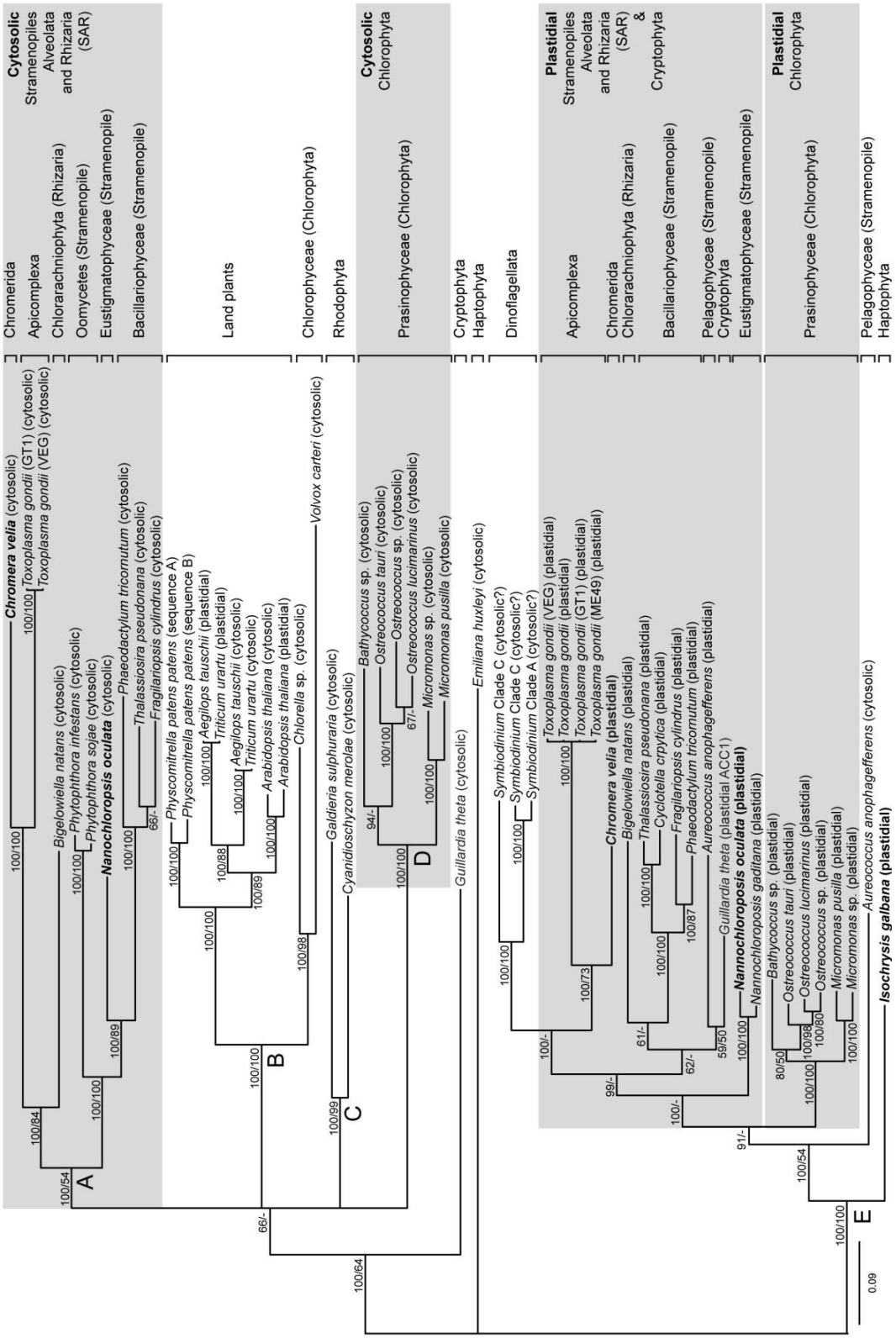

Fig. 3.2 Phylogenetic consensus tree (MrBayes; WAG+G) based on 55 ACCase sequences and 564 amino acids positions. Cytosolic and plastidial ACCase are indicated. Sequences produced in this study are shown in bold. Statistical support for internal nodes was determined by Bayesian inference posterior probabilities (first, shown as % values) and bootstrap analysis for ML (second, Model LG+G+F). Only support values ≥50% are shown.

Node A represents a major clade consisting of cytosolic ACCase of the Chromerida, Apicomplexa and Stramenopiles and a Chlorarachniophyte (Rhizaria) (Fig. 3.2). The other two Chromalveolate taxa, *Guillardia theta* (Cryptophyta) and *Emiliana huxleyi* (Haptophyta), form an exception and are located outside of the five nodes. Within the SAR cluster, the relationship between the Chromerid *Chromera velia* and the Apicomplexan *Toxoplasma gondii* is well supported, and the more distant relationship with the Rhizarian *Bigelowiella natans* is identified, while the Stramenopiles form their own sub-clade. Based on these results, the proposed inclusion of the Stramenopiles, Alveolata and Rhizaria as SAR is supported, while the Cryptophyte and the Haptophyte are more distantly related (Burki et al., 2007, Burki et al., 2008). However, ACCase-based phylogenies do not recover the close relationship of the Cryptophyte with the Archaeaplastida.

Node E contains all plastidial sequences of ACCase, with the exception of the land plants. Here *G. theta* (Cryptophyta) is nested strongly within the SAR species. In contrast to the phylogeny of plastidial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Harper and Keeling, 2003), plastidial ACCase of the haptophyte *Isochrysis* aff. *galbana* (TISO) is more basal to the SAR species, which agrees with a recent multi-gene study based on genomic DNA (Burki et al., 2012). Surprisingly, the Prasinophyceae, which contain a plastid derived from a primary endosymbiotic event, are clustering strongly with the Chromalveolates, which contain a plastid derived from either a secondary or tertiary endosymbiotic event (Fig. 3.2). This relationship was also identified using multi-gene analyses in Stramenopiles, Cryptophyta, and Haptophyta (Frommolt et al., 2008, Moustafa et al., 2009), where a strong association of certain genes with the Prasinophyceae has been found. This is in contrast to the true grasses, where an ancestral gene duplication event resulted in the functional expression of a homomeric, plastidial ACCase, coupled with a loss of heteromeric ACCase (Bryant et al., 2011). A separate gene duplication event, similar to the true grasses, occurred in the ancestor of *Arabidopsis thaliana*, resulting in a plastid-targeted copy of homomeric ACCase, which is expressed alongside the heteromeric ACCase, although at very low levels (Bryant et al., 2011).

The close relationship between plastidial ACCases from SAR, Cryptophyta and Prasinophyceae shown here can either be explained by horizontal or endosymbiotic gene transfer, while gene duplication can be excluded as an explanation (Fig. 3.2). This is in contrast to the replacement of the bacterial GAPDH gene found in the plastids of Archaeplastida with a eukaryotic GAPDH gene during the secondary endosymbiotic event that lead to the Apicomplexa, Cryptophyta, Dinoflagellata,

Stramenopiles, and Haptophyta (Fast et al., 2001). The replacement occurred through duplication of the cytosolic GAPDH, as is evident from the close phylogenetic relationship between the cytosolic and plastidial GAPDH in these taxa (Fast et al., 2001). The presence of a cryptic green endosymbiont in the red lineage has been refuted (Archibald, 2009a, Deschamps and Moreira, 2012), which makes an endosymbiotic gene transfer of ACCase unlikely. The strong relationship of the plastidial ACCase between the SAR species and the Prasinophyceae can therefore be explained by horizontal gene transfer from an unidentified organism, which could either be a Prasinophyte or an unsequenced alga related to the Prasinophyceae. The distant relationship of the cytosolic ACCase sequences of the SAR and Prasinophytes, in contrast, indicates that the ancestral hosts were only distantly related (Fig. 3.2). The phylogenetic analysis of ACCase provides additional support for a serial secondary endosymbiotic event that gave rise to the green plastid-containing Chlorarachniophyta (Rhizaria) within the SAR, This requires the loss of the red algal plastid and regain of a green plastid in the Chlorarachniophyta (see Archibald (2009a; 2012)). Even though this is less parsimonious, having acquired a plastid once could make subsequent acquisitions of plastids easier, similar to the case of the dinoflagellates (Gould et al., 2008).

A further point of interest in Node E is the clustering of the plastidial ACCases of *C. velia* and *T. gondii* and the ACCases of *Symbiodinium* (Fig. 3.2). This supports the close relationship of *C. velia* with the Dinoflagellata and Apicomplexa (Moore et al., 2008). Based on the α-CT binding motif, the *Symbiodinium* sequences were identified as cytosolic; however, they cluster strongly with the plastidial sequences of the SAR. Dinoflagellates are known to have complicated nuclear genomes, which could make it difficult to determine the localisation of the ACCase. Given the close relationship of *C. velia* and apicomplexan parasites, plastidial ACCase could be a potential target for drug development. *Chromera velia* could therefore be used as a substitute to screen compounds for the treatment of apicomplexan parasites, since it is easier to cultivate as is not dependent on a host (Moore et al., 2008). ACCase inhibitors, often based on commercial herbicides acting on plastidial ACCase in the true grasses, have been investigated as potential drugs to treat apicomplexan infections and have shown promise in the reduction of the parasite load (Gornicki, 2003, Zuther et al., 1999). However, not all inhibitors showed the same activity (Louie et al., 2010). The latter could be due to differences in the presence, localisation and expression of ACCase between different species of apicomplexan parasites making a "one-size-fits-all" solution unlikely (MacRae et al., 2012). Furthermore, apicomplexan parasites are only

dependent on *de novo* synthesis of FAs during their liver life stage (schizonts), while trophozoites (blood life cycle stage) are able to access plasma TAGs to supplement their FA needs (MacRae et al., 2012, Bazzani et al., 2012), therefore limiting FA synthesis-based treatments to the liver life stage.

Node B is well supported and consists of the cytosolic and plastidial ACCase sequences of land plants and the cytosolic sequences of green algae (Fig. 3.2). Within the land plants, the two sequences of the moss *Physcomitrella patens patens* form a close relationship. Furthermore, the plastidial and cytosolic ACCases of *Arabidopsis thaliana* form their own sub-clade, while the plastidial and cytosolic ACCaces of the true grasses (*Triticum urartu* and *Aegilops tauschii*) are clearly separated from each other, as well as from the sequences of *A. thaliana*. This demonstrates that the plastidial and cytosolic ACCases in land plants are paralogous within the true grasses and also within *A. thaliana*. Finally, the well supported node C consists of the cytosolic sequences of the two red algal species, while node D shows a single clade consisting of the cytosolic ACCase of the more ancient marine green Prasinophyceae (Fig. 3.2).

4.3 Heteromeric acetyl-CoA carboxylase

To investigate the phylogenetic relationship of β -carboxyltransferase (β-CT) between different taxa, a phylogenetic consensus tree was reconstructed using Bayesian Inference and Maximum Likelihood methods (Fig. 3.3). The general topology of both trees was the same, showing three major nodes (A, B and C), split into four clades (1 to 4). The three major nodes (A to C) were well supported through BI and ML (Fig. 3.3). Clade 3 showed the strongest support for all internal nodes, followed by clades 2 and 4. Clade 1 was not as strongly supported and shows generally poor resolution, with the exception of three species that cluster well together (*Dunaliella salina, Chlamydomonas reinhardtii* and *Volvox carteri*).

The most interesting finding is the strong support for clade 4, made up of cyanobacteria and the Rhizaria *Paulinella chromatophora* (Fig. 3.3), providing additional support for a second independent and more recent primary endosymbiotic event (Marin et al., 2005, Nakayama and Ishida, 2009). Until recently, the primary endosymbiosis of a cyanobacterium was considered to only have occurred once, leading to the primary endosymbiotic plastid containing land plants, Glaucocystophytes and red and green algae. The fact that all four subunits of the heteromeric ACCase are found in the chromatophore (plastid) genome of *P. chromatophora* (Nowack et al., 2008), while the plastid genome of all other heteromeric ACCase containing photosynthetic eukaryotes only encodes for the β-CT subunit further supports this hypothesis.

Clade 2 provides good statistical support for the close relationship between the ancestral Charophyta and derived embryophytes, providing additional evidence for the existence of a common ancestor of the Charophyta and land plants (Timme et al., 2012). The Charophyta/land plant clade branches with the sister clade 1, containing the core Chlorophyta (Chlorophyceae, Ulvophyceae, Trebouxiophyceae). However, the core Chlorophyta clade was not well resolved in this analysis, showing a mixture of branching. This might be an indication that the close relationship within the core Chlorophyta cannot be easily resolved using amino acid sequences and would need the analysis of nucleotide sequences.

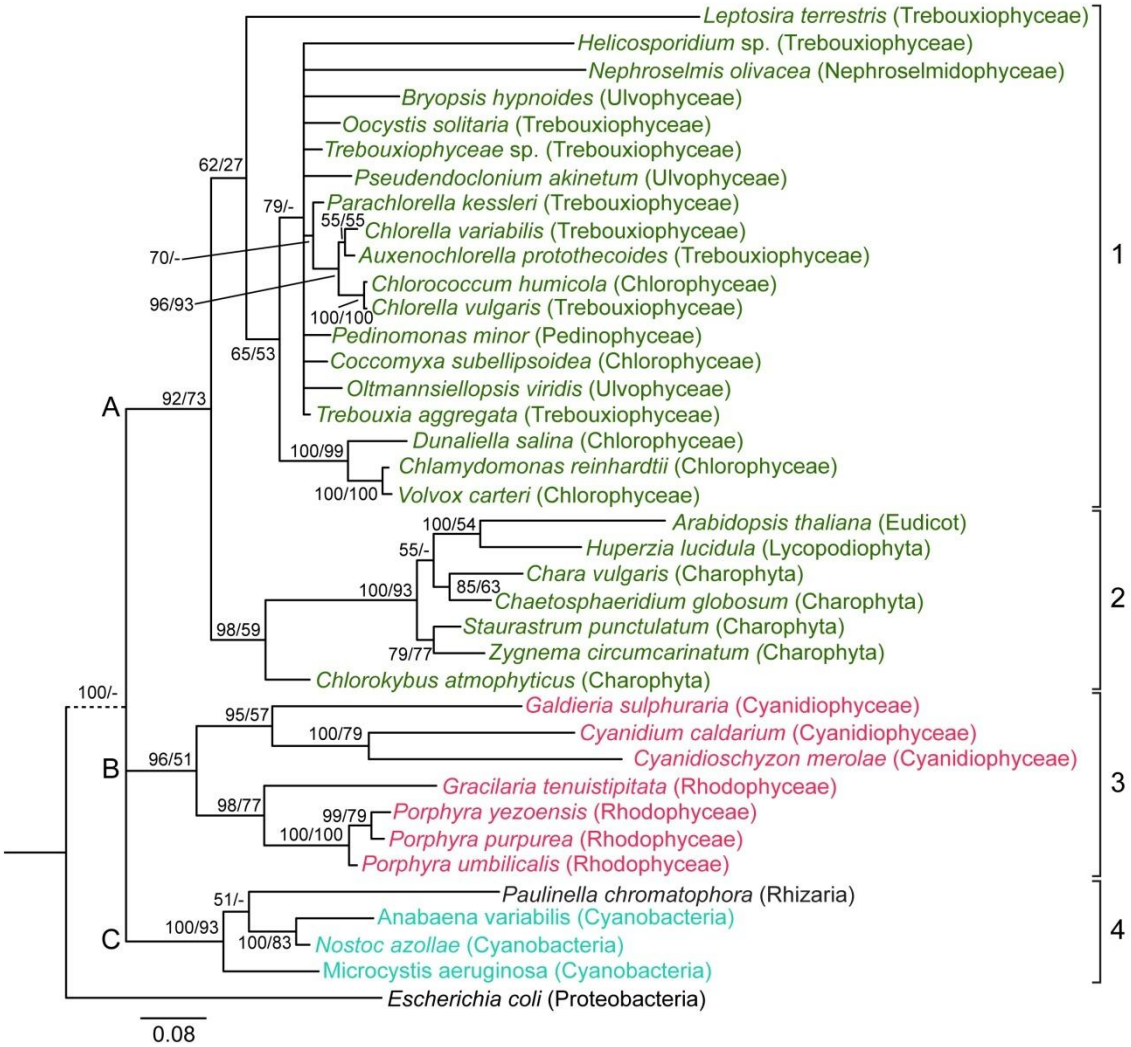

Fig. 3.3 Phylogenetic consensus tree (MrBayes, CpREV) based on 38 β-CT sequences and 213 amino acids positions. The consensus tree includes sequences from green and red microalgae, plants and one rhizaria. Rooted with *Escherichia coli*. Statistical support for internal nodes was determined by Bayesian inference posterior probabilities (first, shown as % values) and bootstrap analysis for ML (second). Only support values ≥50% are shown. Dashed line indicates truncation of branch.

The Prasinophytes are a paraphyletic group of algae consisting of the Nephroselmidophyceae and Mamiellales, among others (Becker and Marin, 2009). The group Mamiellales, of which the genomes of *Osterococcus* spp., *Micromonas* spp. and

Bathycoccus sp. are fully sequenced, are more basal to the remaining algae and contain homomeric ACCase in their plastid. In contrast, the Nephroselmidophyceae *Nephroselmis olivacea* is closer related to the core Chlorophyta and contains heteromeric ACCase in its plastid. The closer relationship is also evident in the clustering of the β-CT subunit sequence with the sequences of the core Chlorophytes in clade 1 (Fig. 3.3).

Lastly, clade 3 consists of the Rhodophytes, showing two nested clades consisting of the Rhodophyceae and the more ancestral Cyanidiophyceae. This nesting is supported by ultra-structural and ecological data, showing the divergence of the Cyanidiophyceae before the endosymbiosis of a heterotrophic eukaryote with a red algae, leading to the red lineage of secondary or tertiary acquired plastids (De Clerck et al., 2012).

4.4 Conclusions

ACCase-based phylogenies can be used to investigate phylogenetic relationships in the highly diverse algae. Expanding on the findings of chapter 2 (Huerlimann and Heimann, 2013), it is demonstrated here that the plastidial homomeric ACCase in the Chromalveolata and Rhizaria was derived from an ancestor or unsequenced relative of the green Prasinophyceae. Why the homomeric ACCase is preferred over the heteromeric ACCase in algae containing a plastid derived from secondary endosymbiosis is unclear. The case of *P. chromatophora* shows that in a primary endosymbiotic event, the heteromeric ACCase is preferred. However, during secondary symbiosis, targeting a single protein (i.e. homomeric ACCase) rather than several subunit peptides (i.e. heteromeric ACCase) to the plastid may be preferred. Alternatively, there could be a general preference of replacing plastid targeted genes present in the ancestor of the endosymbiont during secondary endosymbiosis, if other alternatives to the genes are available. This can be seen in the cases of ACCase (present study), glyceraldehyde-3-phosphate dehydrogenase (Fast et al., 2001) and fructose-1,6-bisphosphate aldolase (Patron et al., 2004) in the investigated SAR species, Haptophyta and Cryptophyta. In the case of ACCase, the plastidial ACCase sequence phylogenies cluster the Prasinophyceae with the SAR group, Haptophyta and Cryptophyta to the exclusion of the cytosolic ACCase, suggesting horizontal gene transfer. Why a new gene was acquired through HGT gene transfer, rather than gene duplication within the host remains to be elucidated.

The distinct lack of genomic data for many Phyla impedes the determination of the origin of genes that have been acquired through horizontal gene transfer. For example, the paraphyletic class Prasinophyceae, which are consistently associated with

Chromalveolates, are currently only represented by the order Mamiellales (*Micromonas* sp., *Ostreococcus* sp. and *Bathyoccus* sp.). Information from the intervening green lineages may reveal that any seemingly unique ancestral acquisition of genes in the Chromalveolata are actually independently derived from multiple HGT events in different Chromalveolate lineages from intervening green lineages which have not yet been sequenced (Elias and Archibald, 2009). As more complete genome sequences become available, the complex picture of algal endosymbiosis will become clearer and the uncertainty about horizontal versus endosymbiotic gene transfer will be resolved for more genes.

5. Acknowledgements

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Chapter 4 - The effect of nitrogen starvation on acetyl-CoA carboxylase expression and fatty acid content in *Chromera velia* and *Isochrysis* aff. *galbana* (TISO)

The following chapter is a collaborative effort of which each author's contribution is outlined below.

Contribution:
Roger Huerlimann: Conception and main execution of project, writing and editing chapter
Eike J. Steinig: Dry weight determination, nutrient analysis
Heather Loxton: Cell counts
Kyall R. Zenger: Supervision, editing
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1. Abstract

Lipids from microalgae have become a valuable product with applications ranging from biofuels to human nutrition. While changes in fatty acid (FA) content and composition under nitrogen starvation are well documented, the molecular mechanisms are poorly understood. Acetyl-CoA carboxylase (ACCase) is a key enzyme in the FA synthesis and elongation pathway. Plastidial and cytosolic ACCases provide malonyl-CoA for *de novo* FA synthesis in the plastid and FA elongation in the endoplasmic reticulum, respectively. The present study aimed at investigating the expression of plastidial and cytosolic ACCase in *Chromera velia* and *Isochrysis* aff. *galbana* (TISO) and their impact on FA content and elongation level when grown under nitrogen-deplete conditions. In *C. velia*, plastidial ACCase was significantly upregulated during nitrogen starvation and with culture age, strongly correlating with increased FA content. Conversely, plastidial ACCase of *I.* aff. *galbana* (TISO) was not differentially expressed in nitrogen-deplete cultures, but upregulated during the logarithmic phase of nitrogen-replete cultures. In contrast to plastidial ACCase, the cytosolic ACCase of *C. velia* was downregulated with culture age and nitrogen-starvation, strongly correlating with an increase in short-chain FAs. In conclusion, the expression of plastidial and cytosolic ACCase changed with growth phase and nutrient status in a species-specific manner and nitrogen starvation did not always result in FA accumulation.

2. Introduction

Photosynthetic microalgae present a natural source for a variety of fatty acids (FAs). Over the last 10 years, there has been an increased interest in microalgal lipids. Many microalgal species are high in lipids, which can be turned into several types of biofuels (Kröger and Müller-Langer, 2012). Furthermore, microalgae are renowned to contain very long-chained polyunsaturated fatty acids (VLC-PUFAs, ≥C20), which are more abundant in algae than in plants (Cagliari et al., 2011, Harwood and Guschina, 2009). VLC-PUFAs are mainly associated with membrane lipids (Hulbert et al., 2013), while short-chain FAs (SC-FAs, C14 to C18) are accumulated as triacylglycerides (TAGs) for storage (Fidalgo et al., 1998). Depending on the intended application, different aspects of microalgal lipids are desired. Low value biofuels depend on high lipid productivities to become economically viable (Chisti, 2013) and short-chain saturated or monounsaturated FAs are preferred (Islam et al., 2013, Stansell et al., 2012). Conversely, VLC-PUFAs are of special interest as essential food additives in the form of ω-3 FAs for human health (Milledge, 2011), and many aquaculture species (Guedes and Malcata, 2012, Hemaiswarya et al., 2011). In contrast to biofuels, these high-value essential food additives are less dependent on high lipid productivity, but on the ratio in abundance of ω-3 VLC-PUFAs like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), compared to ω-6 FAs, e.g. linoleic acid (LA) and arachidonic acid (ARA) (Ryckebosch et al., 2012, Simopoulos, 2006).

Algae growing under stress conditions, such as high intensity light (Van Wagenen et al., 2012), high temperature (Van Wagenen et al., 2012), high salinity (Renaud and Parry, 1994) and phosphate or nitrogen starvation (Breuer et al., 2012, Fidalgo et al., 1998, Liang et al., 2012), often exhibit an increase in FA content, which potentially can be applied to improve lipid productivities for commercial applications (Chisti, 2013). Conversely, stress conditions are typically negatively correlated with biomass productivities, as energy and carbon use is diverted to storage products and possibly production of molecules for stress protection (Courchesne et al., 2009, Hu et al., 2008). Increases in lipid content, however, typically result in reduced VLC-PUFA content, with FAs decreasing in length and desaturation level, and an increase in SC-FAs associated with TAGs (Dunstan et al., 1993, Fidalgo et al., 1998, Grima et al., 1992, Huerlimann et al., 2010). While these biochemical changes in algae are well documented, molecular mechanisms driving lipid accumulation and the decrease in VLC-PUFAs are poorly understood and data on the expression of genes involved in lipid synthesis is lacking (Hu et al., 2008).

The lipid synthesis pathway in algae is thought to be closely related to that of plants (Cagliari et al., 2011, Huerlimann and Heimann, 2013). Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) is a key enzyme in the lipid synthesis pathway and catalyses the formation of malonyl-CoA from acetyl-CoA in the plastid (chloroplast) and the cytosol of plants and algae (Huerlimann and Heimann, 2013). Plastidial-derived malonyl-CoA is then used by fatty acid synthase (FAS) for the *de novo* synthesis of FAs, while cytosol-derived malonyl-CoA is used for elongation of FAs in the endoplasmic reticulum (Huerlimann and Heimann, 2013). This ultimately yields VLC-PUFAs generated by desaturases and elongases in the endoplasmic reticulum (Baud et al., 2003). Since plastidial ACCase provides malonyl-CoA for *de novo* FA synthesis, we expect its expression to be upregulated during lipid accumulation caused by nitrogen starvation. Conversely, we expect cytosolic ACCase to be upregulated during nitrogen-replete conditions, providing malonyl-CoA for the elongation of VLC-PUFAs, which are highly abundant in actively growing cultures. Therefore, it is important to distinguish between the two possible localisations of ACCase, since they are expected to behave differently at different culture stages (culture age).

There are two forms of plastidial ACCase, depending on the origin of the plastid. Most algal groups with a plastid derived from primary endosymbiosis contain nuclear-encoded, heteromeric plastidial ACCase, consisting of four separate subunits (biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and α- and β-carboxyltransferase (α -CT and β -CT)), while algae with plastids derived from a secondary or tertiary endosymbiotic event contain homomeric plastidial ACCase consisting of one long, multifunctional enzyme (see chapter 2 and Huerlimann and Heimann (2013) for details). Measuring the expression of a single subunit of the heteromeric ACCase might not reflect the expression of the remaining three subunits and therefore the activity of the enzyme. In contrast, gene expression analyses of homomeric ACCase directly measure the abundance of the only transcript needed for the production of functional ACCase.

One of the earliest series of experiments on homomeric ACCase demonstrated a strong correlation between ACCase content, activity and FA synthesis rate in *Isochrysis galbana* (Sukenik and Livne, 1991). However, this study did not differentiate between cytosolic and plastidial ACCase. Knowledge of plastidial and cytosolic ACCase expression patterns in different species at various growth phases and/or under varying growth conditions is important for manipulating microalgal cultures for either improved lipid productivity for biofuel production or improving yields of ω-3 VLC-PUFAs for nutritional supplements. In the last 10 years, several studies investigated

the expression of ACCase in algae. Two studies measured gene expression of different forms of ACCase directly through real-time quantitative PCR (Lei et al., 2012, Wan et al., 2011), while three transcriptome studies explored genes generally involved in lipid synthesis, including heteromeric or homomeric ACCase (Cheng et al., 2013, Liang and Jiang, 2013, Rismani-Yazdi et al., 2012). While all studies showed a stress-related upregulation of the heteromeric ACCase subunit or homomeric ACCase, respectively, four studies also showed a positive correlation between gene expression and FA content (Lei et al., 2012, Liang and Jiang, 2013, Rismani-Yazdi et al., 2012, Wan et al., 2011), and one study did not measure FA content (Cheng et al., 2013). Furthermore, two studies also reported a decrease in expression of cytosolic ACCase under stress conditions (Rismani-Yazdi et al., 2012, Wan et al., 2011).

These studies demonstrated that ACCase gene expression in algae changes with culture age and nutrient status, but the interaction between the two was not investigated. Investigating the combination of nutrient stress and growth phases will provide a better understanding on how algae react to nutrient starvation over time, critical knowledge required for improved industrial biofuel and/or nutrient supplement production. Furthermore, algae comprise a wide variety of different phyla with different biochemical properties, and the regulation of the FA synthesis pathway might vary considerably between organisms. Therefore more research is required to cover more taxonomic groups.

The aim of the present study was to investigate the gene expression of plastidial ACCase in *Isochrysis* aff. *galbana* (TISO), an important aquaculture species, and *Chromera velia* (a new marine species of a new order bridging the evolutionary gap between the obligate apicomplexan parasites and dinoflagellates (Moore et al., 2008)) during logarithmic, late logarithmic and stationary growth phases under nitrogen-deplete and nitrogen-replete conditions. Gene expression was then correlated with FA accumulation in the different growth phases. Furthermore, we also correlated the expression of cytosolic ACCase with FA elongation in *C. velia*.

3. Materials and Methods

3.1 Algal culture

Isochrysis aff. *galbana* (CS-177, TISO strain, NQAIF001) was obtained from the CSIRO Marine Laboratories in Hobart, Tasmania, Australia, and *Chromera velia* (NQAIF136) was isolated from Sydney Harbour, New South Wales, Australia (Moore et al., 2008). All species are kept in the culture collection of the North Queensland Algal Identification / Culturing Facility (NQAIF) at James Cook University, Townsville, Australia. The algae were cultured in outer reef ocean seawater (Orpheus Island, Great Barrier Reef, Queensland, Australia), that was filtered through a membrane (Millipore, Durapore PVDF 0.45 µm, WH PL 47mm) and autoclaved.

3.2 Experimental culture set-up

Four cultures per species were first grown under nitrogen- and phosphate-replete conditions until a cell concentration of approximately 1.0 x 10^7 to 1.2 x 10^7 cells mL^{-1}, coinciding with the depletion of nitrate and nitrite in the culture medium. The four replicate cultures were pooled and divided into eight 2 L culture flasks at an inoculation density of 4 x 10^6 cells mL^{-1}. All cultures were supplied with standard L1-medium (Andersen et al., 2005), with the following exceptions: 1) all cultures contained twice the amount of NaH_2PO_4 H_2O (0.725 x 10^{-4} M) to prevent phosphate limitation and cultures were supplied with additional phosphate as appropriate; 2) only the four replicate N+ cultures (nitrogen-replete) were supplied with $NaNO_3$ (8.82 x 10^{-4} M), while the four replicate N- cultures (nitrogen-deplete) where not supplemented with any nitrogen.

Cultures were maintained at 28 °C, a light-dark cycle of 12:12 h, and an average photon flux intensity of 138 µmol m^{-2} s^{-1} provided by cool white fluorescent lights. Cultures were aerated individually with air filtered through a 0.45 µm filter (Durapore; Millipore). Replicate cultures were placed in two rows of four bottles, with alternating N- and N+ treatments, and rotated clockwise by three positions on a daily basis. Cell density and nutrient samples were taken every day at 8 am at the onset of the light phase. Samples for dry weight, total lipid and gene expression were taken during the logarithmic (L), late logarithmic (LL) and stationary phase (S).

3.3 Cell density and ash-free dry weight determination

Cell density was determined in quadruplicates with a Neubauer-improved haemocytometer. Dry weight concentration was determined as described by Lamers et al. (2010). Ammonium formate (0.5 M, pH 8) was used as washing buffer. Samples were dried to constant weight in 10 mL beakers over 48 hours at 100 °C for dry biomass weight determinations. This was followed by 12 hours at 500 °C to determine the weight of the ash component. Ash-free dry weight (AFDW) was calculated as the difference between dry biomass weight and the weight of the ash component.

3.4 Harvesting method for total lipids and fatty acid profiles

The following volumes were harvested in the respective phases by centrifugation at 3,000 x g for 20 min at 20 °C (Beckman Coulter, Avanti J-26 XPI): *Isochrysis*: L: 500 mL, LL 2: 400 mL, S 200 mL. *Chromera velia*: L 400 mL, LL 400 mL, S 400 mL. The pellets were resuspended in 30 mL ammonium formate (0.5 M, pH 8), transferred into a 50 mL tube and centrifuged at 3,000 × g for 20 min at 20 °C (Eppendorf, 5810R). After the supernatant was removed, the pellets were frozen at -80°C, freeze-dried for 24 h (VirTis benchtop 2K, VWR) and stored at -80°C.

3.5 Total lipids

Total lipids were determined gravimetrically following a direct extraction and transesterification method adapted from Bligh and Dyer (1959) by using lyophilised biomass. The method was adapted by changing the solvent system to less toxic solvents; benzene, toluene and chloroform have been replaced with hexane (Cohen et al., 1988, Lewis et al., 2000, Rodríguez-Ruiz et al., 1998, von Alvensleben et al., 2013). Total lipid values were corrected for ash content and are expressed in mg g^{-1} AFDW or % of AFDW throughout this manuscript.

3.6 Fatty acid profile

Fatty acids were extracted and directly transesterificated in situ using methanol:acetylchloride (95:5 (v:v)), as described in Gosch et al. (2012) following the method adapted from Cohen et al. (1988) and Rodríguez-Ruiz et al. (1998). The fatty acid methyl esters (FAME) were separated and quantified by gas chromatography – mass spectrophotometry (GC/MS) (Agilent 7890 GC with FID – Agilent 5975C EI/TurboMS, Agilent Technologies Australia Pty Ltd). The GC temperature parameter was programmed following David et al. (2002), with a gradient from 50 to 250 °C. FAME peaks were identified by comparison of relative retention time data with a FAME

reference standard (FAME mix C4-C24; Sigma Aldrich, Australia) and further confirmed using mass-spectrometry data together with the NIST08 Mass Spectral Library. A comparison of peak areas with authentic external standards (Sigma Aldrich) was used to quantify the FAMEs. Recovery was calculated using nonadecanoic acid (C19:0) as the internal standard. Individual FAME results were corrected for ash content and are expressed in [mg g-1 AFDW] or [% of AFDW]. Total fatty acid content was determined as the sum of all FAMEs.

3.7 Total RNA extraction

At harvest, a 10 mL sample was taken for each replicate and centrifuged at 3,000 x g for 10 min at 20 °C (Beckman Coulter, Allegra X12R). The cell densities of all samples were adjusted to a total of 5 x 10^6 cells for the extraction in sterile RNase-free tubes and centrifuged a second time at 3,000 x g for 10 min at 20 °C (Beckman Coulter, Microfuge 22R). First, 100 µL of 0.8 mm Zirconium Oxide beads (Next Advance, Lomb Scientific, Taren Point NSW, Australia) and then 200 µL of Ultraspec RNA extraction reagent (Fisher Biotec, Wembley WA, Australia) were added to the samples. The samples were then homogenised for 5 min at intensity 9 in a BBX24B-CE Bullet Blender Blue (Next Advance, Lomb Scientific). Another 300 µL of Ultraspec reagent was added to all samples, bringing the volume up to 500 µL. RNA extractions were carried out with the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine CA, USA.) according to the manufacturer's recommendations with a total volume of 500 µL of Ultraspec reagent and 500 µL ethanol. The DNase treatment was carried out with 80 µL of DNase I cocktail and 50 µL of RNase-free water was used to elute. The quality of the RNA was confirmed with a post-gel stained 1% agarose gel and spectrophotometry (NanoDrop spectrophotometer, BIOLAB Analytical Technologies) was used to determine concentration, 230 nm/260 nm and 260 nm/280 nm ratios. Samples were stored at -80 °C until cDNA synthesis.

3.8 Quantitative real-time PCR

First strand synthesis was carried out from total RNA (1 µg for *I.* aff. *galbana* (TISO) and 0.5 µg for *C. velia*) using iScript Reverse Transcription Supermix for RT-qPCR (Order # 1708841, Bio-Rad) in 20 µL total volume. Primers (Table 4.1) were designed using PRIMER3 (Rozen and Skaletsky, 1999) in Geneious (V6.0, Drummond et al. (2011)) with a fragment length between 75 and 200 base pairs, and synthesised by GeneWorks (http://www.geneworks.com.au). The quality of the primers was assessed using PerlPrimer (V1.1.21, Marshall (2004)) and Net Primer (http://www.premierbiosoft.com/netprimer/). Primers were chosen to contain the least

amount of extensible and non-extensible primer dimer, hair-pins, repeats and palindromes. ACCase primers designed based on sequences presented in chapter 3. Reference genes were identified specifically for this study from the same transcriptome, as has been described for the ACCase sequences in chapter 3 (Table 4.2).

Table 4.1 Primer sequences, melting temperature (Tm) and amplified fragment length of target and reference gene of *C. velia* and *I.* aff. *galbana* (TISO). Plastidial ACCase is abbreviated ACC1, while cytosolic ACCase is abbreviated ACC2.

Species	Gene	Purpose	Primer name	Sequence (5' to 3')	Tm [°C]	Fragment length [bp]
C. velia	ACC1	Target	C04-F	CACTGAAGAAGACGCAAAGGG	54	131
			C04-R	GGTTCTCCACTCCGATAGACAG	57	
	ACC2	Target	C09-F	GCCACGAACGATACAGCACTCAA	57	127
			C09-R	ACACCGCACCCTCAGACAGAAC	58	
	GAPDH	Reference	C13-F	GGTCGTCTCCAACGCTTCTT	54	163
			C13-R	TCTCCAGTCCTTGCCTCCCT	56	
	TUB	Reference	C18-F	CATCAAGTCCTCTGTCTGCG	54	185
			C18-R	CTGTGAACTCCATCTCGTCC	54	
I. aff. galbana (TISO)	ACC1	Target	B02-F	CCAATACGAACAACTACGCCAAC	55	78
			B02-R	CGACAAAGACGGCATCCACA	54	
	GAPDH	Reference	B11-F	AGGGCGGTGCTAAGAAGGTC	56	114
			B11-R	AGGCGTTGGAGAGGATGTTGA	54	
	TUA	Reference	B14-F	AAGCGTGCCTTCGTCCACTG	56	107
			B14-R	CCAACCTCCTCGTAGTCCTTCTC	59	
	TUB	Reference	B16-F	TGTTGTCCGTAAGGAGGCTG	54	110
			B16-R	TCTTGGAGATGAGAAGGGTGC	54	

The best reference genes for the conditions in the present experiment (*C. velia:* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-tubulin (TUB)*, I.* aff. *galbana* (TISO): GAPDH, alpha-tubulin and TUB) were determined using GeNorm (Vandesompele et al., 2002) in the qBASE program (Version: 2.5, Hellemans et al. (2007)). Sequence data from this article can be found in the GenBank data libraries under accession numbers: ACCase KF673095 to KF673099, reference genes KF953487 to KF953491. qPCR was carried out using a CAS-1200 Automated Sample Setup system (Corbett Robotics) with a Rotor-Gene 6000 (Corbet Robotics) thermocycler in a 100-well disk format (Qiagen) using SsoFast EvaGreen supermix (Bio-Rad) for detection. Reactions contained 5 µL sample (equals 1.25 ng template based on initial RNA concentration), 0.6 µL of each primer (final concentration 400 nM), 1.3 µL dH_20 and 7.5 µL supermix for a total reaction volume of 15 µL. Cycle parameters were 30 s at 95 °C for denaturation and 25 s at 55 °C for annealing/elongation. Melting curves were routinely checked for abnormalities. Ct values and amplification efficiencies were calculated in LinRegPCR (version 2013.0, Ruijter et al. (2009)). Three biological and three technical replicates were performed for each sample and relative expression was determined based on multiple reference genes using qBase. The results presented in this report are normalised to the condition with the lowest expression level, which was set at 1.

Table 4.2 Identification of reference genes

Species	NCBI accession #	Closest match on Genbank, % identity	E-value	NCBI accession #	Defined function
C. velia	KF953487	glyceraldehyde-3-phosphate dehydrogenase *C. velia* (100%)	0	ACF48281	GAPDH
	KF953488	beta-tubulin *Moneuplotes crassus* (97.4%)	0	P20365	TUB
I. aff. galbana (TISO)	KF953489	glyceraldehyde-3-phosphate dehydrogenase *I. galbana* (91.4%)	0	AAQ63753	GAPDH
	KF953490	alpha-tubulin *Chloromonas* sp. ANT3 (89.1%)	0	AAB86648	TUA
	KF953491	beta-tubulin *Emiliania huxleyi* CCMP1516 (99.3%)	0	EOD11672	TUB

4. Results

4.1 Nutrient analysis

Nitrogen-containing culture media of *I.* aff. *galbana* (TISO) and *C. velia* were nitrogen-deplete on day 7 and on day 3, respectively. *Chromera velia* consumed phosphate at a much higher rate than *I.* aff. *galbana* (TISO), but adequate levels of phosphate were maintained for both species at all times and phosphate concentrations were never below 1 mg L^{-1} PO_4^{3-}, preventing the possibility of phosphate limitation. Build-up of media nitrite through incomplete reduction of nitrate and secretion was negligible for both species (below 1 mg L^{-1} NO_2^-).

4.2 Growth and lipid content

Isochrysis aff. *galbana* (TISO) generally showed slower growth and reached an overall lower cell density and ash-free dry weight (AFDW) (N-: 7.02 x 10^6 cells mL^{-1}, 0.400 g L^{-1}; N+: 1.13 x 10^7 cells mL^{-1}, 0.574 g L^{-1}) than *Chromera velia* (N-: 1.14 x 10^7 cells mL^{-1}, 0.876 g L^{-1}; N+: 1.76 x 10^7 cells mL^{-1}, 0.960 g L^{-1}) (Fig. 4.1). *Chromera velia* exhibited similar growth under nitrogen-replete and nitrogen–deplete conditions until day 4 for cell densities and until day 8 for biomass (AFDW). In contrast, cell densities and biomass differed between nitrogen-replete and nitrogen–deplete conditions for *I.* aff. *galbana* (TISO) from day 1 (Fig. 4.1).

Overall, *I.* aff. *galbana* (TISO) displayed no change in FA and total lipid contents with growth phase and between nitrogen treatments (Fig. 4.1). In contrast, *C. velia* exhibited a 2.8-fold increase in FA content and a 1.9-fold increase in total lipid content between the logarithmic phase of nitrogen-replete cultures and the stationary phase of nutrient-deplete cultures. The FA content of *I.* aff. *galbana* (TISO) showed a significant interaction between growth phases and nitrogen treatment ($F_{2,12}$: 7, P=0.0111) and differences between growth phases were close to being significant (mean values: L 11.1 %, LL 11.7 % S 11.2 %; $F_{2,12}$: 4, P=0.0623), but there was no significant difference between nitrogen treatments (mean values: N- 11.2 %, N+ 11.5 %; $F_{1,6}$: 1, P=0.3400). However, observed differences in FA content of *I.* aff. *galbana* (TISO) between treatments were too small to be significant at a biological level, and can thus be

considered constant. Conversely, the FA content of *C. velia* ranged from 8.3 % (N+, L) to 23.6 % (N-, S) and was significantly higher in nitrogen-deplete cultures than in the nitrogen-replete cultures (mean values: N- 18.0 %, N+ 11.0%; $F_{1,6}$: 1891, P<0.0001) and in later growth phases (mean values: L 10.6 %, LL 13.6 % S 19.2%; $F_{2,12}$: 994, P<0.0001). Additionally, there was a significant interaction between nitrogen treatment and growth phase ($F_{2,12}$: 66, P<0.0001).

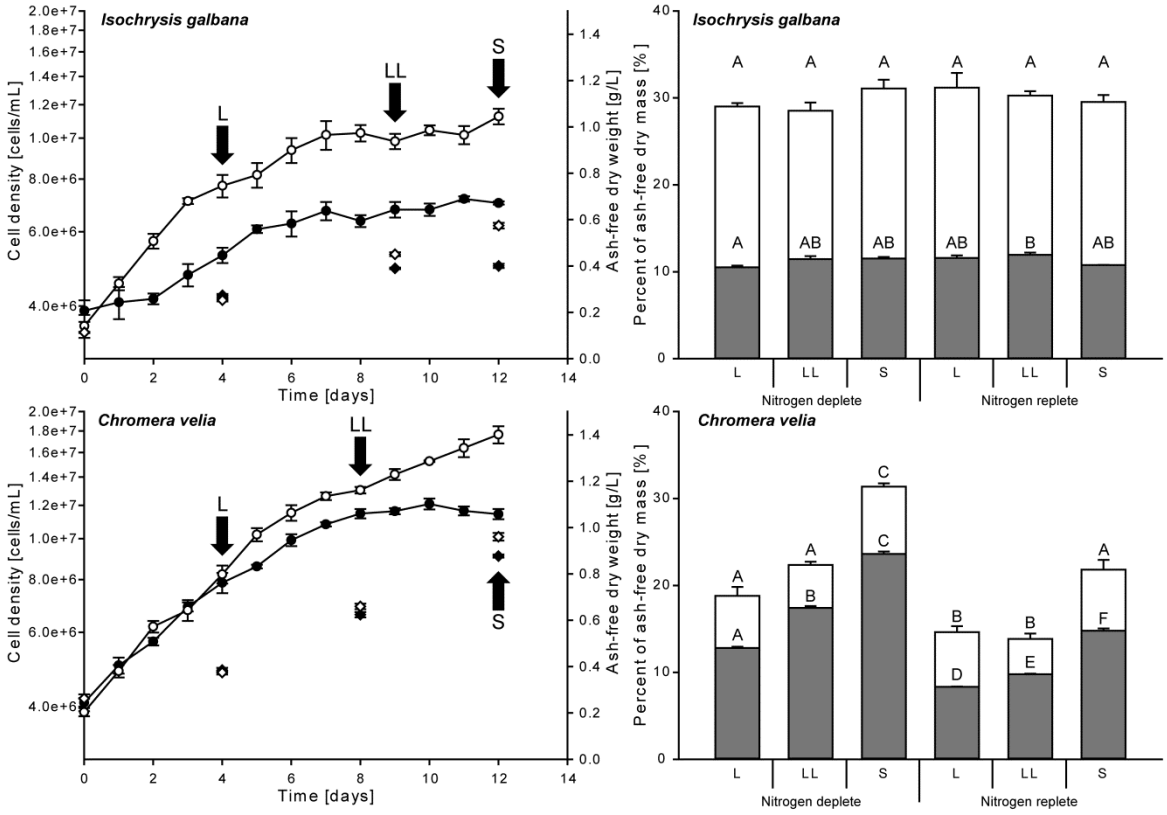

Fig. 4.1 Left: Cell density (circles) and ash-free dry weight (diamonds) of nitrogen-deplete (closed) and nitrogen-replete (open) cultures of *Isochrysis* aff. *galbana* (TISO) and *Chromera velia*. Mean ± SE is shown, n = 4. Arrows indicate where cultures were harvested (L = logarithmic phase, LL = late logarithmic phase, S = stationary phase). Right: FAME (grey), non-FAME lipids (white) and total lipids (sum of grey and white) content of ash-free dry biomass for nitrogen-deplete and -replete cultures at different harvesting times. Mean ± SE is shown, n = 4.

Total lipid content of *I.* aff. *galbana* (TISO) did not differ significantly between nitrogen treatments and growth phases, and contained on average 19 % of AFDW non-FA lipids (Fig. 4.1). In contrast, the total lipid content of *C. velia* increased significantly in later growth phases (mean values: L 17.7 %, LL 18.0 %, S 25.5 %; $F_{2,12}$=66, P<0.0001), and was significantly higher in nitrogen-deplete cultures compared to nitrogen-replete cultures (mean values: N- 24.2 %, N+ 16.5 %; $F_{1,6}$=149, P<0.0001) and there was a significant interaction between growth and nitrogen treatment ($F_{2,12}$=6.6, P=0.0114). In contrast to *I.* aff. *galbana* (TISO), the non-FA fraction was much smaller for *C. velia* (on

average 6 % of AFDW) and the observed increase in total lipid content was mainly attributable to an increase in the FAME fraction.

AFDWs differed considerably more in *I.* aff. *galbana* (TISO), but less in *C. velia* (Fig. 4.1). This is especially the case during the stationary phase (difference in AFDW: *I.* aff. *galbana* (TISO) 43.5 %, *C. velia* 9.6 %), whereas the difference in cell density of *C. velia* is similar to *I.* aff. *galbana* (TISO). The smaller difference in AFDWs in *C. velia* can be explained by the increased accumulation of lipids in nitrogen-deplete cultures in stationary phase, leading to larger cells. Based on these data, we expect an upregulation of plastidial ACC1 in *C. velia* in the later growth phases and with nitrogen-starvation, while there should be no upregulation in *I.* aff. *galbana* (TISO).

4.3 Plastidial ACCase

Chromera velia showed a 4-fold upregulation of expression of plastidial ACCase in nitrogen-deplete cultures during late logarithmic and stationary growth phases, while the corresponding upregulation was only approximately 2-fold in nitrogen-replete cultures (Fig. 4.2). There was a significant difference among treatment ($F_{1,4}$=125, P=0.0004), growth phase ($F_{2,8}$=117, P<0.0001) and the interaction between both ($F_{2,8}$=25, P=0.0003). Furthermore, the expression of plastidial ACCase showed a significant positive correlation with FAME content (r = 0.83, $t_{(2)16}$ = 5.99, P < 0.001). This indicates an important role for plastidial ACCase in the production of FAs during the later growth phases of nitrogen-stressed cultures of *C. velia*.

Expression of plastidial ACCase in *I.* aff. *galbana* (TISO) was strongly upregulated during logarithmic (4-fold) and stationary (5.5-fold) growth phases in nitrogen-replete cultures (Fig. 4.2). Conversely, in nitrogen-deplete cultures there was no significant effect of growth phase on expression. Statistically, there was a significant difference with treatment ($F_{1,4}$=33, P=0.0046), growth phase ($F_{2,8}$=74, P<0.0001) and an interaction between both ($F_{2,8}$=28, P=0.0002). In contrast to *C. velia*, the expression of plastidial ACCase in *I.* aff. *galbana* (TISO) showed no significant correlation with FA content (r = -0.25, $t_{(2)16}$ = 1.03, P = 0.3165). Upregulation of plastidial ACCase in the logarithmic phase of *I.* aff. *galbana* (TISO) in nitrogen-replete cultures is contrary to FA-based predictions of expression, but can be explained by having to maintain a FA content of approximately 10% during this period of highest growth, which is effectively halved at each cell division.

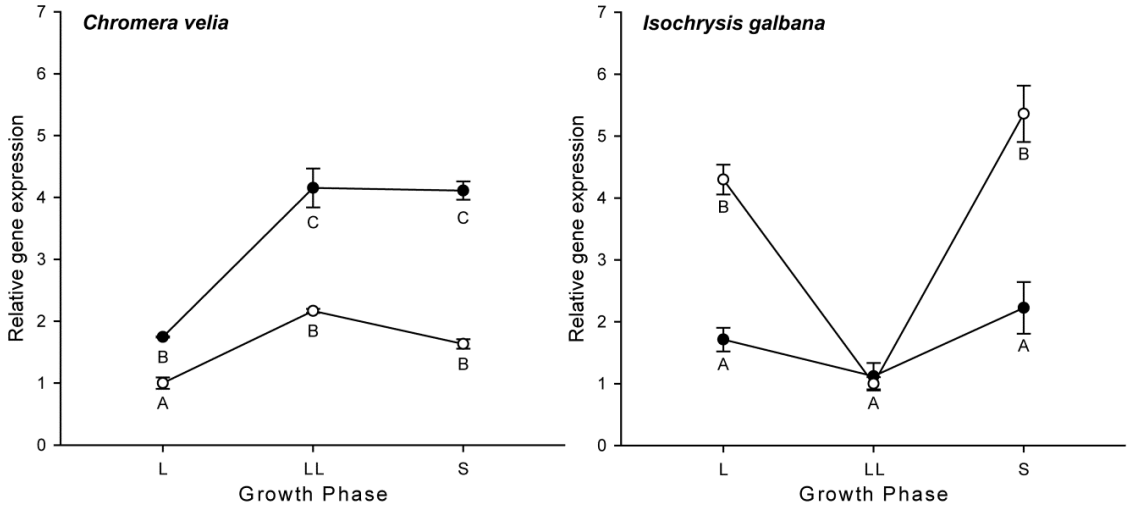

Fig. 4.2 Relative gene expression of plastidial ACCase in nitrogen-deplete (closed) and nitrogen-replete (open) cultures of *C. velia* (left) and *I.* aff. *galbana* (TISO) (right). Mean ± SE is shown, n = 3. Letters indicate significant differences based on Tukey's post-hoc test.

4.4 Cytosolic ACCase and fatty acid composition in *Chromera velia*

In *C. velia*, the average amount of short chain fatty acids (SC-FA, length ≤ C18) was significantly higher in later growth phases (mean values: L 61.2 mg g^{-1}, LL 92.9 mg g^{-1}, S 146.4 mg g^{-1}; $F_{2,12}$=1627, P<0.0001) and in nitrogen-deplete cultures (mean values: N- 131.6 mg g^{-1}, N+ 68.8 mg g^{-1}; $F_{1,6}$=1909, P<0.0001), and there was a significant interaction between growth phase and nitrogen treatment ($F_{2,12}$=58, P<0.0001) (Fig. 4.3). The amount of very long-chain fatty acids (VLC-FA, length ≥ C20) differed significantly between nitrogen treatment (mean values: N- 48.0 mg g^{-1}, N+ 40.9 mg g^{-1}; $F_{1,6}$=138, P<0.0001), increasing slightly in nitrogen-deplete cultures (from L: 44.5 mg g^{-1} to S: 52.0 mg g^{-1}), while it decreased slightly in nitrogen-replete cultures (from L: 44.4 mg g^{-1} to S: 39.4 mg g^{-1}) (Fig. 4.3). There was also a significant difference with growth phase (mean values: L 44.5 mg g^{-1}, LL 43.1 mg g^{-1}, S 45.7 mg g^{-1}; $F_{2,12}$=6, P=0.0142) and a significant interaction between growth phase and nitrogen treatment ($F_{2,12}$=37, P<0.0001) was also observed. Changes in VLC-FA content, however, were minor compared to those observed for SC-FA content.

Since cytosolic ACCase provides malonyl-CoA for the elongation of fatty acids, we expected gene expression of cytosolic ACCase to decrease with culture age and be lower in the nitrogen-deplete cultures. As expected, the relative expression of cytosolic ACCase decreased 2- to 4-fold with progressing culture age and was lower in nitrogen-deplete cultures. Statistically, there was a significant difference with nitrogen treatment ($F_{1,4}$=9.6, P=0.0364), growth phase ($F_{2,8}$=117, P=0.0009), but no interaction between the two ($F_{2,8}$=25, P=0.6303) was observed. The expression of cytosolic ACCase

showed a significant negative correlation with short chain fatty acid (≤ C18) content (r = -0.78, $t_{(2)16}$ = -5.02, P<0.0001), while correlations with VLC-FA content were not significant (≥ C20) (r = -0.37, $t_{(2)16}$ = -1.62, P=0.1258). Even though a slight increase in VLC-FAs was observed in nitrogen-deplete compared to nitrogen–replete cultures, gene expression of cytosolic ACCase was not significantly different in logarithmic and late logarithmic growth phases (Fig. 4.3). Thus, the observed slight increase in VLC-FA is most likely explained by an overall increase in FA content in nitrogen-deplete cultures, especially FAs of ≤ C18 and extending to FAs of length ≥ C20. The lack of a significant correlation between the expression of cytosolic ACCase and VLC-FAs mirrors the behaviour of plastidial ACCase in *I.* aff. *galbana* (TISO). During logarithmic growth, *de novo* FA synthesis produces FAs with a length of C18, which in turn are elongated in the ER to FAs of a length ≥ C20. This allows maintenance of a required level of VLC-FAs during logarithmic growth. When cell division slows with culture age, membrane VLC-FAs require less replenishment and the expression of cytosolic ACCase is downregulated, thereby possibly allowing SC-FAs (≤ C18) to accumulate.

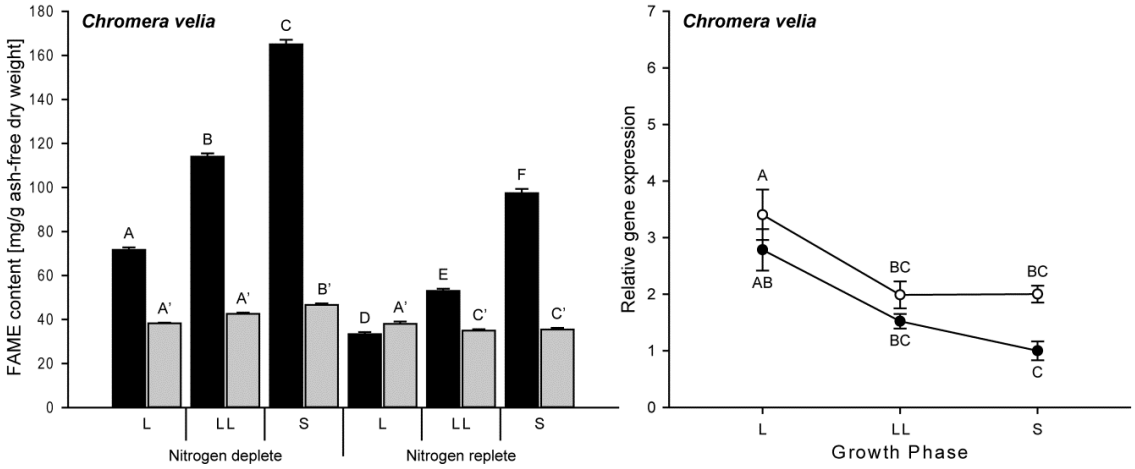

Fig. 4.3 Left: FAME content divided into FA of ≤C18 length (black) and ≥C20 length (grey) for *Chromera velia*. Mean ± SE is shown, n = 4. Right: Relative gene expression of cytosolic ACCase in nitrogen-deplete (closed) and nitrogen-replete (open) cultures of *C. velia*. Mean ± SE is shown with n = 4 for FAME content and n = 3 for gene expression. Statistically significant differences are indicated by different letters. Prime letters are used for FA of ≥C20 length (grey).

5. Discussion

Many algae accumulate fatty acids (FAs) in the form of triacylglycerides (TAGs) when cell division slows due to certain stress conditions such as high light-intensity (Van Wagenen et al., 2012), high temperature (Van Wagenen et al., 2012), high salinity (Renaud and Parry, 1994) and phosphate or nitrogen starvation (Breuer et al., 2012, Fidalgo et al., 1998, Liang et al., 2012). This increase in FA content is a desirable outcome for commercial FA-based product development. Despite the importance to improve FA or TAG yields, the relationship between the expression of genes involved in FA synthesis and effects on FA content are still poorly understood. Investigating the combination of nutrient stress and growth phase (culture age) provides a better understanding on how algae react to nutrient starvation with culture age, which is important information for the nascent microalgal industry. The present study investigated the gene expression of plastidial and cytosolic homomeric ACCase in the haptophyte *Isochrysis* aff. *galbana* (TISO) and the Chromerida *Chromera velia* to fill this knowledge gap. We demonstrated that the two species reacted different to nitrogen starvation and culture age in terms of ACCase expression and FAME content.

In *C. velia*, the increased expression of plastidial ACCase correlated positively with increasing FAME content over time, which agrees with the findings of other studies (Lei et al., 2012, Liang and Jiang, 2013). Furthermore, there was a strong interaction between nitrogen status and growth phase, where nitrogen-deplete cultures responded to nitrogen starvation with an increased expression of plastidial ACCase. Culture nitrogen status was also important, as nitrogen-replete cultures showed a weaker response with regards to lipid and FAME accumulation than cultures set up under nitrogen-deplete conditions. The interaction between growth phase and nutrient status has important implications for industrial production of this species and others that exhibit similar response patterns, since nitrogen status at the beginning of the culture period influences expression levels of genes involved in FA synthesis and therefore accumulation.

Previous studies demonstrated that the amount of long-chain fatty acids (VLC-FAs, length ≥ C20) decreases under nutrient starvation and increasing culture age while the amount of short-chain fatty acids (SC-FAs, length ≤ C18) increases (Dunstan et al., 1993, Grima et al., 1992, Huerlimann et al., 2010). Here I demonstrated that cytosolic ACCase was downregulated with culture age, explaining the mechanisms of observed increases in SC-FAs, as cytosolic ACCase provides malonyl-CoA for the elongation of existing SC-FAs, which are the building blocks for the formation of VLC-FAs in the

inter-membrane space of the endoplasmic reticulum. Consequently, starving algal cultures of nitrogen or cultivation to stationary phase will potentially reduce the yield of valuable VLC-PUFAs. Thus for the production of VLC-FAs, like EPA and DHA, nitrogen status and growth phase are important considerations for industries targeting these markets.

To overcome shortages of VLC-PUFAs production for human consumption, the genetic modification of higher biomass yielding organisms, such as oleaginous fungi, yeast and plants has been proposed (Cao et al., 2012, Certik and Shimizu, 1999, Graham et al., 2007). Unfortunately, VLC-FA productivity in plants is still low, with elongase substrates being one of the potential bottlenecks (Graham et al., 2007). In this context, available pools of malonyl-CoA for FA elongation could be an additional bottleneck, explaining the low yields in these studies. It might therefore be necessary to overexpress cytosolic ACCase alongside with appropriate elongases and desaturases to produce higher quantities of VLC-PUFAs.

Interestingly, not all algae react to nitrogen starvation with accumulation of FAs. The FAME content of *I.* aff. *galbana* (TISO), a commercial DHA producer, did not vary significantly between growth phases nor between nitrogen-replete and nitrogen-deplete cultures, which differs from results presented by Fidalgo *et al.* (1998). The seemingly contradictory results, however, can be explained by strain-specific responses, as they used *I. galbana* Parke, whereas this study investigated the strain *I.* aff. *galbana* (TISO). While both strains contain high levels of DHA, only the Parke strain contains high levels of EPA and differences in optimal growth temperatures have also been reported (Liu and Lin, 2001).

Gene expression of plastidial ACCase in *I.* aff. *galbana* (TISO) was generally significantly lower in nitrogen-deplete cultures and was not upregulated between growth phases, in contrast to responses observed in logarithmic and stationary phase in nitrogen-replete cultures. The low ACCase expression in nitrogen-deplete cultures is consistent with results from the earliest experiments on ACCase content and activity in *I. galbana*, where nitrogen refertilisation of nitrogen-deplete cultures resulted in an increase in ACCase content and activity (Livne and Sukenik, 1992). Given the nitrogen-induced differences in growth patterns between nitrogen-replete and nitrogen-deplete cultures, a significant difference in FAME or lipid content would be expected, considering actively growing cultures halve their FAME and lipid content with each division. The increase in plastidial ACCase expression in nitrogen-replete cultures of *I.* aff. *galbana* (TISO) during the logarithmic growth phase is a response to the increased

growth demand of VLC-PUFAs for maintaining membrane integrity of the actively dividing cultures, allowing the cells to maintain adequate levels of membrane FAs. It is thus not surprising that the increased expression of plastidial ACCase during the logarithmic growth phase was not observed in the nitrogen-limited cultures, which grew considerably slower. In addition, the Δ4-, Δ5- and Δ8- desaturases of *I.* aff. *galbana* (TISO) were upregulated in logarithmic phase of cultures grown in nitrogen-replete conditions as presented in chapter 5 of this thesis. This upregulation of expression corresponded to an increase in PUFAs in actively growing cells. Together, this shows that actively dividing cultures of *I.* aff. *galbana* (TISO) upregulate ACCase and desaturases to facilitate the production of membrane lipids, maintaining high levels of PUFAs.

In contrast, the increase in expression of plastidial ACCase during the stationary phase of nitrogen-replete cultures is not as easily explained. A similar upregulation was observed in stationary phase of nitrogen-replete cultures for Δ4-, Δ5- and Δ8-desaturases of *I.* aff. *galbana* (TISO), which also did not correlate with an increase PUFAs as discussed in chapter 5. Diversion of malonyl-CoA for production of carotenoids or sterols appears to be unlikely for two reasons: 1) carotenoids and steroids are made from isoprenoids, which are not made from malonyl-CoA and 2) carotenoids and steroids are generally lipid-soluble and would therefore be a constituent of the total lipid fraction, leading to an expected increase of the total lipid content in stationary phase. A more likely explanation for the observed increase in plastidial ACCase without concomitant increase in FAME and FAs would be that malonyl-CoA may be used as a substrate for polyketide synthesis. In plants, chalcone synthase uses paracoumaroyl-CoA and malonyl-CoA to produce chalcones and flavonoids, which function in protection from UV exposure (Paiva, 2000). Similar compounds could potentially also function in algae in response to environmental stress. Unfortunately, polyketide synthesis pathways are not well studied in algae (Cardozo et al., 2007). Another possible explanation for the observed stationary phase results would be immediate recycling of produced FAs through β-oxidation, to build other compounds, not resulting in enhanced growth, counteracting the nitrogen starvation and utilisation of the photosynthetically-acquired carbon.

In summary, we demonstrated that expression of plastidial and cytosolic ACCase and FAME accumulation and composition are species-dependent, changing with growth phase and nitrogen status, but affecting the investigated species in different ways. Considering the close phylogenetic relationship of *I.* aff. *galbana* (TISO) and *I. galbana* Parke, and their varied responses to nitrogen starvation, future research should

compare the effects of nitrogen starvation on plastidial and cytosolic ACCase expression and downstream effects on FAME content and composition of the two species. Additionally, as demonstrated by the species-specific responses in plastidial and cytosolic ACCase expression, studies should be expanded to investigate responses of different algal phyla of commercial importance, as responses can greatly affect development of cultivation conditions (e.g. fertilisation regime and harvest alignment with growth phase) for improved end-product development. Lastly, further research should aim at measuring FA content and composition in different types of lipids (e.g. storage TAG and membrane phospho- and glycolipids) to assess the compartmentalisation of FAs.

Chapter 5 - Effects of growth phase and nitrogen starvation on expression of fatty acid desaturases and fatty acid composition of *Isochrysis* aff. *galbana* (TISO)

The following chapter is a collaborative effort of which each author's contribution is outlined below.

Contribution:
Roger Huerlimann: Conception and execution of project, writing and editing chapter
Eike J. Steinig: Dry weight determination, nutrient analysis
Heather Loxton: Cell counts, support with culturing
Kyall R. Zenger: Supervision, editing
Dean R. Jerry: Supervision, editing
Kirsten Heimann: Supervision, editing

This chapter has been prepared as a publication and is under review at *Gene* with the authors: Roger Huerlimann, Eike J. Steinig, Heather Loxton, Kyall R. Zenger, Dean R. Jerry and Kirsten Heimann

1. Abstract

Very long-chain polyunsaturated fatty acids (VLC-PUFAs) are important dietary requirements for maintaining human health. Many marine microalgae are naturally high in ω-3 VLC-PUFAs; however, the molecular mechanisms underpinning fatty acid (FA) desaturation and elongation in algae are poorly understood. An advanced molecular understanding might facilitate improvements of this nascent industry. This chapter aimed at investigating expression responses of four front-end fatty acid desaturase genes and downstream effects on FA profiles to nitrogen starvation and cultivation growth stage in *I.* aff. *galbana* (TISO). Cultures were grown in nitrogen-replete and -deplete medium; samples were harvested during logarithmic, late logarithmic and stationary growth phases to analyse FA content/composition and gene expression of Δ^6-, Δ^8-, Δ^5- and Δ^4-desaturases (d6FAD, d8FAD, d5FAD and d4FAD, respectively). d6FAD exhibited no differential expression, while d8FAD, d5FAD and d4FAD were significantly upregulated during logarithmic growth of nutrient-replete cultures, coinciding with rapid cell division. In conclusion, it is demonstrated that expression of some FADs in *Isochrysis* aff. *galbana* (TISO) varies with culture age and nitrogen status which has downstream consequences on FA desaturation levels. This has implications for the commercial production of VLC-PUFAs where a trade-off between total lipid yield and VLC-PUFAs has to be made.

2. Introduction

Very long-chain polyunsaturated fatty acids (VLC-PUFAs, length ≥ C20) play a key role in the proper membrane function of organisms (Hulbert et al., 2013), but also work as precursors for a variety of signalling molecules in animals (e.g. eicosanoids (Rowley et al., 2005)). Of particular interest are the VLC-PUFAs arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which are of importance not only for human health (Das et al., 2012), but also the rearing of many aquaculture species like fish, crustaceans and molluscs (Guedes and Malcata, 2012).

In humans, ARA, EPA and DHA are imperative for the development and functioning of the brain, reduction of inflammatory responses and cardiovascular health (Khozin-Goldberg and Cohen, 2011, Lenihan-Geels et al., 2013, Ward and Singh, 2005). The dependence on these fatty acids (FAs) is exacerbated by the low efficiency of animals to produce VLC-PUFAs, which decreases even more with age (Khozin-Goldberg and Cohen, 2011). Due to this low production efficiency, coupled with their immense importance in human health, ARA, EPA and DHA are now increasingly considered to be essential nutrients (Khozin-Goldberg et al., 2011). It is therefore important to supply ARA, EPA and DHA in sufficient quantities through diet. For example, early infant cognition depends on sufficient supplies of ARA and DHA (Qawasmi et al., 2012), which are now routinely added to infant formulae (Khozin-Goldberg et al., 2011). Many modern chronic diseases (e.g. cardiovascular diseases, cancer and autoimmune diseases) are aggravated by high ω-6 to ω-3 essential fatty acids ratios of 10-20:1 in Western diets (Simopoulos, 2008). Recent research has shown that ratios of below 6:1 have many health benefits (Gomez Candela et al., 2011, Simopoulos, 2008). Consequently, it is important to supply the right kind of fatty acids (FA) in correct proportions; however, environmentally sustainable supply of VLC-PUFAs remains a challenge (Adarme-Vega et al., 2014).

Traditionally, the main source of EPA and DHA has been fish oil from wild-caught fatty fish, such as mackerel or salmon (Ward and Singh, 2005), which has the additional advantage of typically lower contents of ω-6 FAs compared to ω-3 FAs. However, over 80 % of the world's fisheries are fully or over-exploited (Frid and Paramor, 2012), and therefore cannot sustainably fulfil increasing demands. An additional problem is the accumulation of organic toxins and toxic metal compounds in wild-caught fish (Meujo and Hamann, 2013). Farmed fish and shellfish, an alternative to wild-caught fish, have become increasingly more important to meet demands and dietary health requirements

by the growing population. As with humans, due to the low capacity of marine animals to naturally produce DHA and EPA, these FAs need to be supplied with their diet. Therefore, the aquaculture industry is still heavily dependent on wild-caught fish to provide adequate amounts of VLC-PUFAs for optimal growth, in addition to proteins (Adarme-Vega et al., 2014). While advances in aquaculture have made it possible to replace the protein part of the diet with plant material (Olsen and Hasan, 2012), viable alternative sources for VLC-PUFAs remain expensive.

As most plants do not contain relevant amounts of EPA and DHA, but are rich in short-chain (SC) PUFAs, especially the essential α-linoleic acid (ALA, C18:3n-3), plant-based diets are inadequate for meeting the requirements for VLC-PUFAs for human health and aquaculture animal production (Lenihan-Geels et al., 2013). While it has been shown that humans and fish can produce EPA from short-chain PUFAs (SC-PUFAs) (especially stearidonic acid (SDA, C18:4n-3)), conversion rates, especially to DHA, are too low to meet dietary requirements (Harris et al., 2008, Petrie and Singh, 2011, Tocher et al., 2006).

Research into genetically modified plants with the appropriate desaturases to produce VLC-PUFAs is still ongoing (Adarme-Vega et al., 2014, Petrie et al., 2012, Petrie and Singh, 2011) and is a complicated process since the complete FA synthesis pathway contains several enzymes (Fig. 5.1). While the production of up to 25% of EPA of the total FA content is possible in plants (Cheng et al., 2010), producing large quantities of DHA proved to be more difficult (Meesapyodsuk and Qiu, 2012, Petrie and Singh, 2011). In a recent breakthrough, Petrie et al. (2012) managed to produce up to 13.9% DHA of total FA in genetically modified *Arabidopsis thaliana*, however, for commercial production this feat would need to be repeated in the crop plant *Brassica napus*, which is used for industrial production of oils. Even though improvements have been made in transgenic plants, there are still many problems to overcome (Adarme-Vega et al., 2014). For example, most transgenic plants are still very high in unwanted ω-6 FAs and a strong resistance to the use of genetically modified organisms persists, especially in Europe and Australia (Venegas-Calerón et al., 2010).

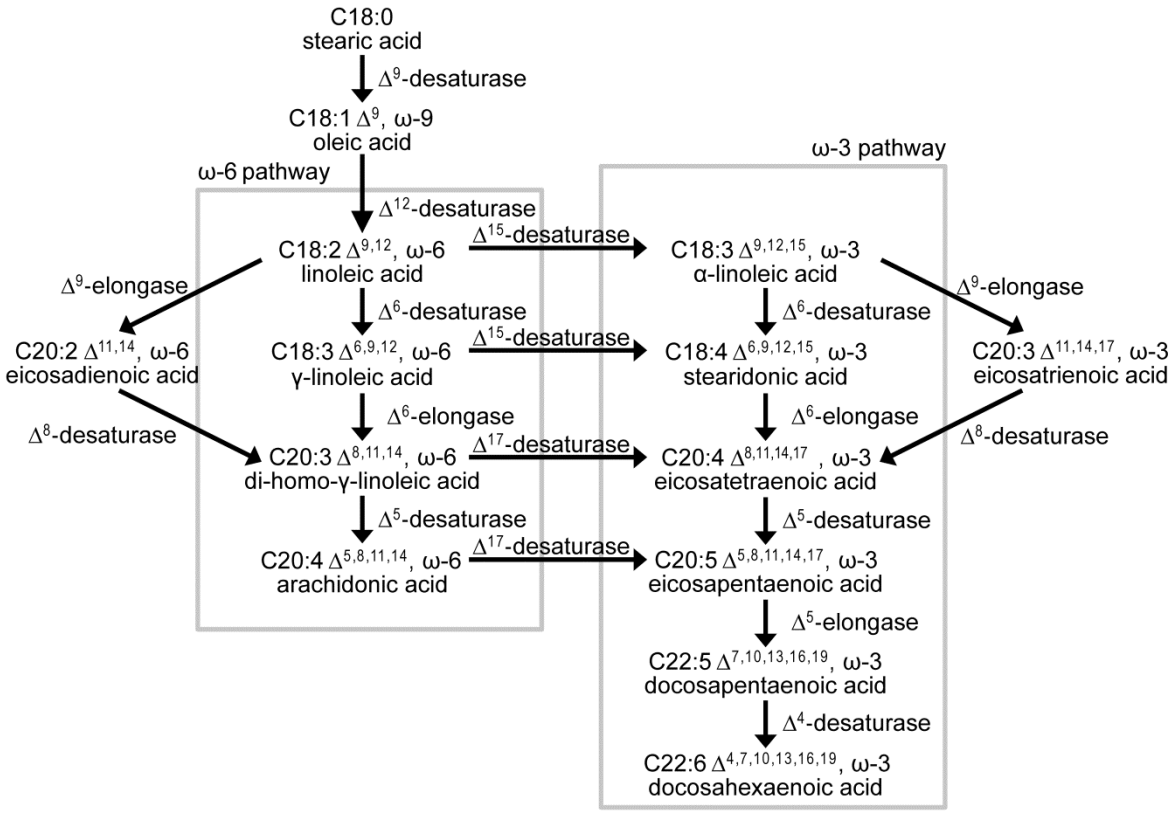

Fig. 5.1 *De novo* synthesis pathways of long-chain polyunsaturated fatty acids in eukaryotic algae (modified from Pereira et al. (2003) and Khozin-Goldberg et al. (2011)).

There is an increasing market for algal VLC-PUFA for human consumption (Khozin-Goldberg and Cohen, 2011). Unlike most plants, some microalgae are naturally high in essential VLC-PUFAs and are the primary source of VLC-PUFAs in marine food webs. For example, *Nannochloropsis oculata* (Huerlimann et al., 2010), *Skeletonema costatum* and *Porphyridium cruentum* (Duong et al., 2012) are rich in EPA; *Isochyrsis* aff. *galbana* (TISO) (Huerlimann et al., 2010) and *Porphyridium cruentum* (Duong et al., 2012) are rich in DHA; while *Pavlova* spp. (Martínez-Fernández et al., 2006) and *Isochrysis galbana* (Parke) (Fidalgo et al., 1998) contain both. Compared to other possible sources, microalgae with a high EPA and DHA content also have potential for high productivities (Adarme-Vega et al., 2012) and are generally low in ω-6 LC PUFA, including ARA (Khozin-Goldberg et al., 2011), however, cost is still a prohibitive problem (Adarme-Vega et al., 2012). A detailed understanding of FA desaturation and elongation pathways can help to estimate the potential of VLC-PUFA productivities in different algal species and open possibilities for the manipulation of FA composition by manipulating environmental conditions (Khozin-Goldberg and Cohen, 2011).

The *de novo* synthesis of FAs in algae (and plants) occurs in the chloroplast, whereas FA elongation and desaturation occurs in the smooth endoplasmic reticulum (ER) (Huerlimann and Heimann, 2013, Khozin-Goldberg and Cohen, 2011). In the plastid,

stearic acid (C18:0), one of the products of *de novo* lipid synthesis, is converted into oleic acid (C18:1n-9) by a Δ^9-desaturase and is exported from the plastid into the smooth ER for further elongation and desaturation. There are two connected parallel pathways in the lipid elongation and desaturation process (Fig. 5.1). The Δ^{12}-, Δ^{15}- and Δ^{17}-desaturases (both ω-desaturases) introduce a double bond between an existing double bond and the acyl end, while the Δ^4-, Δ^5-, Δ^6- and Δ^8-desaturases (all front-end desaturases), introduce a double bond between an existing double bond and the carboxyl end (Hashimoto et al., 2008). The ω-6 pathway starts with linoleic acid (LA, C18:2) and ultimately produces ARA, while the ω-3 pathway starts with ALA and results in EPA and later DHA (Fig. 5.1). Apomorphic animals generally lack Δ^{12}-desaturases, as well as ω-desaturases (i.e. Δ^{15}- and Δ^{17}-desaturases), making linoleic acid and ALA essential (Pereira et al., 2003). Furthermore, the low abundance and rate of Δ^6-desaturase is limiting production of ARA, EPA and DHA from the precursors LA and ALA in humans, animals, plants and yeast (Qi et al., 2004, Ward and Singh, 2005). Humans also lack Δ^4-desaturase, while the low efficiency Δ^6-desaturase is involved in an alternative retro-conversion pathway that includes a controlled β-oxidation step from C24:6n-3 to produce DHA (see Pereira et al. (2004) for detail on this alternative pathway in animals). Certain eukaryotes (*Euglena gracilis* (euglenoid), *Tetrahymena* sp. (ciliate), *Acanthamoeba* (soil amoeba), *Phaeodactylum tricornutum* (stramenopile), *Emiliania huxleyi* and *I. galbana* (haptophytes)) have a mechanism to bypass the low specific reaction rate Δ^6-desaturation/elongation step (Khozin-Goldberg et al., 2011, Pereira et al., 2003), enabling these protists to produce di-homo-γ-linoleic acid (DGLA, C20:3) and eicosatetraenoic acid (ETA, C20:4) from LA and γ-linoleic acid (GLA, C18:3), respectively, through the use of a Δ^9-elongase and a Δ^8-desaturase (Fig. 5.1).

Desaturases have different substrate specificities for FAs bound to a protein (acyl-ACP), a co-factor (acyl-CoA) or being part of a phospholipid (acyl-lipid). In most algae, the investigated desaturases were specific to acyl-lipids, but a few exhibit specificity for acyl-CoA (Meesapyodsuk and Qiu, 2012). The individual front-end desaturases generally show low specificity to a single pathway (ω-6 or ω-3), but can convert FA from either pathway (Ma et al., 2011a, Pereira et al., 2003). For example, the Δ^6-desaturase cloned from *Nannochloropsis oculata* converts LA equally well to GLA as ALA to stearidonic acid (SDA, C18:4n-3) (Ma et al., 2011a). Δ^5- and Δ^6- fatty acid desaturases cloned from *Phaeodacylum tricornutum* also converted the respective FAs from both pathways (Fig. 5.1) (Domergue et al., 2002). However, some appear to be specific to one pathway, for example a newly identified acyl-CoA-specific Δ^6-desaturase shows a preference for ω-3 substrates (Petrie et al., 2010). Considering

that desaturases and elongases generally work in both pathways, it is surprising that many marine algae are unusually high in ω-3 FAs and low in ω-6 FAs. It follows that a specific mechanism is required that favours the ω3-pathway over the ω-6 pathway, which could be achieved by strong activities of ω-15- and ω-17 desaturases, funnelling the ω-6 FAs towards the ω3 pathway (Petrie and Singh, 2011). Alternatively, FAs could be funnelled to pools that cannot be accessed by desaturases and elongases, facilitated by transfer of FAs from glycerophospholipids and acyl-CoA, to which they are bound during desaturation and elongation, to lipid compounds that are not accessible by substrate-specific desaturases and elongases, as observed in plants (Abbadi et al., 2004). This partitioning can explain why some algae can accumulate FAs from different parts of the same pathway. In the last 10 years, there has been an increased focus on researching the desaturation and elongation of fatty acids (FA) in algae. VLC-PUFAs have an important role in the function of cells through their important effect on cell membranes fluidity.

Low temperatures induce the production of membrane PUFAs in some algae, for example in *Nannochloropsis* sp., *Chlorella* sp. (James et al., 1989), *Chlamydomonas* sp. (Zhang et al., 2011), *Pavlova salina* (Zhou et al., 2007) and *Rhodomonas* sp. (Renaud et al., 2002). However, temperature is not the only factor influencing FA content and composition. The FA composition can also change with culture age/growth phase and nutrient status. For example, *N. oculata*., *I.* aff. *galbana* (TISO)*, Tetraselmis* sp, and *Rhodomonas* sp. exhibit a shift from a high proportion of PUFAs in logarithmic phase towards an increased amount of monounsaturated and/or saturated FAs in late logarithmic and stationary phase (Huerlimann et al., 2010). This reduction in PUFAs also leads to a large reduction of VLC-PUFAs, especially EPA in *Nannochloropsis oculata*. Unfortunately, there is still a distinct lack of information on the molecular basis of these biochemical changes.

Previous expression studies in microalgae have investigated the effect of temperature on the expression of Δ^6-desaturase in *N. oculata* (Ma et al., 2011b) and Δ^8-, Δ^6- and Δ^4-desaturases in *Pavlova salina* (Zhou et al., 2007), and the effect of nitrogen starvation on expression of Δ^{12}-, Δ^6- and Δ^5-desaturases in the green microalgae *Parietochloris incisa* (Iskandarov et al., 2010). To date, no study investigated the effect of nitrogen starvation and culture age on expression patterns of all four front end desaturases involved in the pathway leading to DHA. Knowing how algae react to nitrogen starvation at different growth phases will help in choosing the right fertilisation and harvesting conditions to improve VLC-PUFA yields and economic feasibility. As the haptophyte *Isochrysis* aff. *galbana* (TISO) has a high DHA content, the aim of this

study was to investigate the impact of growth phase (logarithmic, late logarithmic and stationary) under nitrogen-replete and nitrogen-deplete conditions on the expression of four front end FA desaturase genes of *I.* aff. *galbana* (TISO).

3. Materials and Methods

Only partial material and methods are presented here. For full length material and methods on "experimental culture set-up", "cell density and ash-free dry weight determination", " harvesting method for total lipids and fatty acid profiles and total RNA", " fatty acid profile", "total RNA extraction", "quantitative PCR" and "statistics" please refer to the material and methods section of chapter 4 of this thesis.

3.1 Algal culture

Isochrysis aff. *galbana* (CS-177, TISO strain) was obtained from the CSIRO Marine Laboratories in Hobart, Tasmania, Australia and is kept in the culture collection of the North Queensland Algal Identification / Culturing Facility (NQAIF) at James Cook University, Townsville, Australia under the accession number NQAIF001. TISO was cultured in L1 fertilised mid to outer reef filtered (Millipore, Durapore PVDF 0.45 µm, WH PL 47mm) and autoclaved seawater (Orpheus Island, Great Barrier Reef, Queensland, Australia).

3.2 Experimental culture set-up

Each treatment consisted of four replicate cultures of *I.* aff. *galbana* (TISO). Aerated cultures were maintained at 28 °C, a light-dark cycle of 12:12 h, and an average photon flux intensity of 138 µmol m^{-2} s^{-1} provided by cool white fluorescent lights. Cell number and nutrient samples were taken every day at 8 am at the onset of the light phase. Samples for ash-free dry weight, total lipid and gene expression were taken during the logarithmic (L), late logarithmic (LL) and stationary phase (S).

3.3 Quantitative real-time PCR

Primers for reference genes were taken from chapter 4 of this thesis, while the sequences of the desaturases were identified specifically for this study (Table 5.1), as described previously in chapter 4. Primers (Table 5.2) were designed using PRIMER3 (Rozen and Skaletsky, 1999) in Geneious (V6.0, Drummond et al. (2011)) with a fragment length between 75 and 200 base pairs, and synthesised by GeneWorks (http://www.geneworks.com.au).

Table 5.1 Identification of genes using a BLASTX search in Geneious (V6.0).

Gene ID	NCBI accession #	nt Length	aa Length	Closest match on Genbank, % identity	E-value	NCBI accession #
d4FAD	KF953492	1,284	427	delta-4 fatty acid desaturase *Isochrysis* sp. GB-2012 (100%)	0	AFJ74710
d5FAD	KF953493	1,320	439	delta-5 fatty acid desaturase *Isochrysis galbana* (100%)	0	AFD22890
d6FAD	KF953494	1,065	354	delta-6 fatty acid desaturase *Isochrysis galbana* (99.4%)	0	AEV77089
d8FAD	KF953495	1,248	415	delta-8 oleate desaturase *Isochrysis galbana* (100%)	0	AFB82640

The best reference genes for the conditions in the present experiment (*C. velia:* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-tubulin (TUB)*, I.* aff. *galbana* (TISO): GAPDH, alpha-tubulin and TUB) were determined using GeNorm (Vandesompele et al., 2002) in the qBASE program (Version: 2.5, Hellemans et al. (2007)). Sequence data from this chapter can be found in the GenBank data libraries under accession numbers: desaturases KF953492 to KF953495, reference genes KF953487 to KF953491. qPCR was carried out using a CAS-1200 Automated Sample Setup system (Corbett Robotics), with a Rotor-Gene 6000 (Corbet Robotics) thermocycler, in a 100-well disk format (Qiagen), using SsoFast EvaGreen supermix (Bio-Rad) for detection. Melting curves were routinely checked for abnormalities. Ct values and amplification efficiencies were calculated in LinRegPCR (version 2013.0 Ruijter et al. (2009)). Three biological and three technical replicates where performed for each sample and relative expression was determined based on multiple reference genes using qBase. The results presented in this report are normalised to the condition with the lowest expression level, which was set at 1.

Table 5.2 Primer sequences, melting temperature (Tm) provided by Geneworks (Thebarton, Adelaide, Australia) and amplified fragment length of target and reference gene of *I.* aff. *galbana* (TISO).

Gene ID	Purpose	Primer name	Sequence (5' to 3')	Orientation	Tm [°C]	Fragement length [bp]
d4FAD	Target	B21-F	GAGAACCTCCGACAAATCCG	Forward	54	119
		B21-R	GTCTGGTAGAGCCCGTTGA	Reverse	53	
d5FAD	Target	B22-F	CGTGAACTGGTGGATGGCTTAC	Forward	57	186
		B22-R	ACGGCGAGGAGATTGACATAC	Reverse	54	
d6FAD	Target	B24-F	CAATGACGAAACGGACGAGTG	Forward	54	98
		B24-R	ACTGGAGCCCGATGTGTAGC	Reverse	56	
d8FAD	Target	B27-F	GCGAATCTAACCTCTACGAGTGC	Forward	57	87
		B27-R	ATTCCACCTTGCGTGCCTT	Reverse	51	
GAPDH	Reference	B11-F	AGGGCGGTGCTAAGAAGGTC	Forward	56	114
		B11-R	AGGCGTTGGAGAGGATGTTGA	Reverse	54	
TUA	Reference	B14-F	AAGCGTGCCTTCGTCCACTG	Forward	56	107
		B14-R	CCAACCTCCTCGTAGTCCTTCTC	Reverse	59	
TUB	Reference	B16-F	TGTTGTCCGTAAGGAGGCTG	Forward	54	110
		B16-R	TCTTGGAGATGAGAAGGGTGC	Reverse	54	

4. Results

4.1 Growth profiles, biomass yields and nutrient status

Detail on growth profiles, biomass yield and nutrient status can be found in the results section of chapter 4 of this thesis (Fig. 4.1). In brief, there was a distinct difference in cell numbers and biomass yield (ash-free dry weight (AFDW)) between *I.* aff. *galbana* (TISO) cultures grown under nitrogen-deplete (N-) and nitrogen-replete (N+) conditions. Differences in cell density were evident from the first day; however, differences in biomass (AFDW) were not apparent until the second harvest on day 9.

4.2 Fatty acid profiles

The total fatty acid (FA) content of *Isochrysis* aff. *galbana* (TISO) ranged from 10.5 to 12.0 % of ash-free dry weigh (Table **5.3**). There was no significant difference between nitrogen treatments ($F_{1,6}$=1, P=0.3400) and growth phases ($F_{2,12}$=4, P=0.0623), but there was a significant interaction between the two factors ($F_{2,12}$=7, P=0.0111). However, in contrast to the FA content, there were considerable shifts in FA composition.

The five major FAs, myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1n-9), stearidonic acid (SDA, C18:4n-3) and docosahexaenoic acid (DHA, C22:6n-3), occurred at concentrations of more than 10 mg g^{-1} AFDW, and accounted for over 70% of the total FAs, independent of treatment and growth phase (Table **5.3**). Minor FAs, occurring at a concentration between 2 and 10 mg g^{-1} AFDW, included palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7), linoleic acid (LA, C18:2n-6) and α-linoleic acid (ALA, C18:3n-3). The remaining FAs occurred at levels < 2 mg g^{-1} AFDW, or were not detected.

Of the 14 main FAs that are part of the ω-3 and ω-6 desaturation and elongation pathways (Fig. 5.1), 11 were identified with the help of the FAME standard (marked in bold in Table **5.3**). The three remaining FAs, eicosatrienoic acid (C20:3n-3), eicosatetraenoic acid (C20:4n-3) and docosapentaenoic acid (C22:5n-3), were not part of the FAME standard. However, no large peaks went unidentified in the chromatograms and it is unlikely that these fatty acids occurred in large quantities. Therefore, even though these important FAs were not included in the FAME standard, they were considered to occur at levels ≤ 1 mg g^{-1} AFDW.

Table 5.3 Fatty acid profiles of *I.* aff. *galbana* (TISO) at logarithmic, late logarithmic and stationary phase grown in nitrogen-replete and deplete cultures. Mean ± SE is shown, n = 4. Fatty acids that are directly part of the ω-3 and ω-6 desaturation and elongation pathways are marked in bold. Letters indicate significant differences based on Tukey's post-hoc test ($p < 0.05$).

		FAME content [mg g^{-1} AFDW]					
		Nitrogen-replete			Nitrogen-deplete		
Fatty Acid	**Common Name**	**L**	**LL**	**S**	**L**	**LL**	**S**
C14:0	Myristic acid	17.8±0.99^{a}	15.7±0.33^{a}	12.3±0.20^{b}	12.4±0.31^{b}	12.5±0.35^{b}	12±0.26^{b}
C14:1n-5	Myristoleic acid	0.2±0.01	0.2±0.00	0.1±0.00	0.1±0.00	0.1±0.00	0.1±0.00
C15:0	Pentadecanoic acid	0.4±0.02	0.4±0.02	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.01
C15:1n-6	Pentadecenoic acid	ND	ND	ND	ND	ND	ND
C16:0	Palmitic acid	12.1±0.55^{a}	13.1±0.31^{ab}	12±0.22^{a}	14.5±0.43^{b}	18.8±0.67^{c}	18.9±0.61^{c}
C16:1n-7	Palmitoleic acid	7.4±0.41^{a}	6.8±0.12^{a}	5.8±0.08^{b}	4.9±0.14^{c}	4.6±0.10^{c}	4.6±0.09^{c}
C16:1n-9	Hypogeic acid	0.2±0.00	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.00	0.2±0.01
C16:2n-4		0.4±0.02	0.4±0.01	0.3±0.00	0.3±0.01	0.2±0.00	0.2±0.00
C16:2n-6		ND	ND	ND	ND	ND	ND
C16:3n-3		ND	ND	ND	ND	ND	ND
C16:3n-4		0.3±0.02	0.3±0.01	0.3±0.00	0.3±0.01	0.3±0.01	0.3±0.01
C16:4n-3		ND	ND	ND	ND	ND	ND
C17:0	Margaric acid	1.1±0.08	0.9±0.03	0.8±0.01	0.7±0.02	0.7±0.01	0.7±0.01
C18:0	**Stearic acid**	**0.1±0.01**	**0.2±0.01**	**0.2±0.01**	**0.2±0.01**	**0.4±0.02**	**0.4±0.02**
C18:1n-7	Vaccenic acid	2.2±0.13^{a}	2.3±0.07^{ab}	2.3±0.06^{ab}	2.2±0.03^{ab}	2.5±0.04^{ab}	2.5±0.05^{b}
C18:1n-9	**Oleic acid**	**8.3±0.59^{a}**	**15.2±0.73^{b}**	**17.2±0.40^{bc}**	**19.2±0.64^{c}**	**28.1±0.92^{d}**	**28.7±0.64^{d}**
C18:2n-6	**Linoleic acid**	**6.0±0.37^{a}**	**6.9±0.22^{b}**	**4.7±0.08^{c}**	**4.7±0.14^{c}**	**4.7±0.19^{c}**	**4.7±0.15^{c}**
C18:3n-3	**α-Linolenic acid**	**9.6±0.43^{a}**	**9.1±0.15^{ab}**	**8.1±0.06^{bc}**	**7.5±0.10^{c}**	**6.1±0.11^{d}**	**5.8±0.12^{d}**
C18:3n-6	**γ-Linolenic acid**	**1.5±0.10^{a}**	**1.6±0.07^{a}**	**1.0±0.02^{b}**	**0.7±0.01^{c}**	**0.4±0.01^{d}**	**0.4±0.02^{d}**
C18:4n-3	**Stearidonic acid**	**34.7±1.56^{a}**	**31.3±0.61^{b}**	**26.9±0.44^{c}**	**22.3±0.38^{d}**	**19.6±0.28^{d}**	**19.6±0.30^{d}**
C20:0	Arachidic acid	0.2±0.02	0.3±0.03	0.4±0.01	0.3±0.04	0.5±0.02	0.6±0.02
C20:1n-11	Gadoleic acid	ND	ND	ND	ND	ND	ND
C20:2n-6	**Eicosadienoic acid**	**0.1±0.00**	**0.2±0.01**	**0.3±0.00**	**0.3±0.01**	**0.4±0.02**	**0.4±0.01**
C20:3n-6	**Dihomo-γ-linolenic acid**	**0.1±0.01**	**0.1±0.01**	**0.2±0.01**	**0.2±0.00**	**0.2±0.01**	**0.2±0.01**
C20:4n-6	**Arachidonic acid**	**0.2±0.01**	**0.2±0.01**	**0.2±0.01**	**0.2±0.00**	**0.2±0.01**	**0.2±0.01**
C20:5n-3	**Eicosapentaenoic acid**	**0.9±0.04**	**1.0±0.04**	**0.8±0.05**	**0.7±0.02**	**0.7±0.01**	**0.8±0.04**
C22:0	Behenic acid	0.2±0.02	0.4±0.03	0.7±0.02	0.7±0.01	1.0±0.06	0.9±0.05
C22:1n-9	Erucic acid	0.1±0.01	0.2±0.03	0.3±0.02	0.4±0.01	0.5±0.05	0.5±0.01
C22:2n-6	Brassic acid	ND	ND	ND	ND	ND	ND
C22:6n-3	**Docosahexaenoic acid**	**12.0±0.15^{a}**	**12.4±0.18^{a}**	**12.4±0.31^{a}**	**11.9±0.08^{a}**	**11.6±0.11^{a}**	**12.3±0.21^{a}**
C24:0	Lignoceric acid	ND	ND	ND	ND	ND	ND
C24:1n-9	Nervonic acid	ND	ND	ND	ND	ND	ND
Total saturated FAs [mg g^{-1} AFDW]		32.0±1.65^{ab}	31.1±0.71^{ab}	26.7±0.34^{c}	29.2±0.74^{bc}	34.2±1.14^{a}	33.8±0.97^{a}
Total monounsaturated FAs [mg g^{-1} AFDW]		18.5±1.14^{a}	24.9±0.86^{b}	26.0±0.51^{b}	27.0±0.67^{b}	35.9±1.11^{c}	36.6±0.76^{c}
Total polyunsaturated FAs [mg g^{-1} AFDW]		65.8±2.28^{a}	63.5±1.13^{a}	55.1±0.74^{b}	49.0±0.51^{c}	44.4±0.68^{c}	45±0.78^{c}
Total ω-3 FAs [mg g^{-1} AFDW]		57.2±1.96^{a}	53.8±0.84^{a}	48.2±0.79^{b}	42.4±0.50^{c}	38.0±0.48^{c}	38.6±0.60^{c}
Total ω-6 FAs [mg g^{-1} AFDW]		7.9±0.48^{a}	9.1±0.31^{a}	6.3±0.10^{b}	6.1±0.16^{b}	5.9±0.23^{b}	5.9±0.18^{b}
Ratio of ω-3/ω-6		7.3±0.34^{ab}	5.9±0.12^{c}	7.7±0.21^{a}	7.0±0.22^{ab}	6.4±0.20^{bc}	6.5±0.12^{bc}
% FAs of AFDW [%]		11.6±0.50^{ab}	12.0±0.24^{b}	10.8±0.05^{ab}	10.5±0.18^{a}	11.5±0.29^{ab}	11.5±0.25^{ab}

SDA was the dominant FA in all growth phases of nitrogen-replete cultures (ranging from 26.9 to 34.7 mg g^{-1} AFDW) and in the logarithmic phase of nitrogen-deplete cultures (22.3 mg g^{-1} AFDW) (Table **5.3**). In contrast, oleic acid was the dominant FA in late logarithmic and stationary phases of nitrogen-deplete cultures (28.1 and 28.7 mg g^{-1} AFDW, respectively). Of the five major and four minor FAs, myristic acid, palmitoleic acid, LA, ALA and SDA exhibited higher concentrations in nitrogen-replete cultures and during logarithmic growth, vaccenic acid and DHA contents did not change with treatment or growth phase, and palmitic acid and oleic acid reached higher amounts in nitrogen-deplete cultures and in stationary phase (Table **5.3**). From this it can be concluded that there is a shift from one group of FAs, including myristic acid, palmitoleic acid, LA, ALA and SDA, to a second group of FAs including palmitic acid and oleic acid, with progressing culture age and nitrogen starvation. Of the two commercially important VLC-PUFAs, EPA and DHA, only DHA was present in larger quantities, ranging from 11.6 to 12.0 mg g^{-1} AFDW, while EPA was only found at ≤ 1.0 mg g^{-1} AFDW. Surprisingly, the DHA content showed no significant difference between growth phases (mean values: L 11.9 mg g^{-1}, LL 12.0 mg g^{-1}, S 12.4 mg g^{-1}; $F_{2,12}$=4, P=0.0636) and between treatments (mean values: N+ 12.3 mg g^{-1}, N- 11.9 mg g^{-1}; $F_{1,6}$=3, P=0.1203), with no significant interaction between growth phases and treatment ($F_{2,12}$=4, P=0.0634). Based on the shift of FA from LA, ALA and SDA to palmitic acid and oleic acid, it can be expected that the relevant desaturase genes are upregulated during logarithmic phase of the nitrogen-replete cultures.

There was also a steady increase in the amount of monounsaturated FAs from the logarithmic phase of nutrient-replete cultures and nitrogen-deplete cultures to the stationary phase, from 18.5 to 26.0 mg g^{-1} AFDW and 27.0 to 36.6 mg g^{-1} AFDW, respectively (Table **5.3**). Overall, there was a significant increase in monounsaturated FAs (MUFAs) with culture age ($F_{2,12}$=72, P<0.0001) and nutrient starvation ($F_{1,6}$=145, P<0.0001), but no interaction was observed between the two factors ($F_{2,12}$=1, P=0.2768). This general increase in monounsaturated FAs was mainly due to increasing amounts of oleic acid (C18:1n-9), significantly increasing with culture age (mean values: L 13.8 mg g^{-1}, LL 21.7 mg g^{-1}, S 23.0 mg g^{-1}; $F_{2,12}$=143, P<0.0001) and nutrient starvation (mean values: N+ 13.6 mg g^{-1}, N- 25.3 mg g^{-1}; $F_{1,6}$=313, P<0.0001). Oleic acid is a direct product of plastidial *de novo* FA synthesis and also provides the substrate for the desaturation and elongation pathway in the endoplasmic reticulum (Fig. 5.1). Therefore, the increase in oleic acid can be explained in two ways: 1) increased plastidial production, or 2) decreased production of polyunsaturated FAs (PUFAs) in the endoplasmic reticulum.

Concomitant with the increase in MUFAs, there was a significant decrease in PUFAs with culture age ($F_{2,12}$=20, P=0.0002) and nutrient starvation ($F_{1,6}$=251, P<0.0001), and a significant interaction between the two factors was observed ($F_{2,12}$=8, P=0.0067). The highest PUFA levels were observed during logarithmic growth of nitrogen-replete cultures with 65.8 mg g^{-1} AFDW, which steadily decreased to 55.1 mg g^{-1} AFDW and was lowest in stationary phase of nitrogen-deplete cultures (45 mg g^{-1} AFDW). The observed decrease in polyunsaturated FAs was due to strong declines of stearidonic acid (SDA, C18:4n-3) levels with culture age (mean values: L 28.5 mg g^{-1}, LL 25.4 mg g^{-1}, S 23.3 mg g^{-1}; $F_{2,12}$=28, P<0.0001) and in nitrogen-deplete cultures (mean values: N+ 31.0 mg g^{-1}, N- 20.5 mg g^{-1}; $F_{1,6}$=254, P<0.0001), and there was an interaction between the two factors ($F_{2,12}$=8, P=0.0070). In addition, LA and ALA showed similar reduction with nitrogen starvation and in later growth phases (Table **5.3**). Considering that there was no change in total FA content, irrespective of fertilisation regime and culture age, it can be concluded that the increase in MUFA content was the result of decreased production of PUFAs.

Isochrysis aff. *galbana* (TISO) contained 5.9 to 7.7 times more ω-3 FA than ω-6 FA, independent of growth phase and treatment. This suggests that the ω-3 pathway is more active than the ω-6 pathway. The bias for ω-3 FA can be explained by differences in substrate specificity of the involved desaturases, and/or the Δ^{15}- and Δ^{17}-desaturases that connect the two pathways could be more active, diverting FAs towards the ω-3 pathway (Fig. 5.1). Overall contents of both ω-3 FAs and ω-6 FA decreased with nutrient starvation, with ω-3 FAs being significantly lower in the nitrogen-deplete cultures (mean values: N+ 53.1 mg g^{-1}, N- 39.7 mg g^{-1} AFDW; $F_{1,6}$=268, P<0.0001) and later growth phases (mean values: L 49.8 mg g^{-1}, LL 45.9 mg g^{-1}, S 43.4 mg g^{-1} AFDW; $F_{2,12}$=21, P=0.0001), and there was also an interaction between growth phase and treatment ($F_{2,12}$=6, P=0.0214) (Table **5.3**). This was mainly due to the decrease of stearidonic acid as discussed above. Based on the decrease in polyunsaturated FAs, it can be expected that the desaturases investigated in this study are upregulated in the logarithmic phase of nitrogen-replete cultures, while being downregulated with culture age and nutrient starvation.

4.3 Gene expression

All four measured fatty acid desaturases (FADs) can potentially take part in both the ω-3 and ω-6 desaturation pathways (Fig. 5.1). Of the four FADs, Δ^8-desaturase (d8FAD), Δ^5-desaturase (d5FAD) and Δ^4-desaturase (d4FAD) showed a similar expression profile, while the Δ^6- fatty acid desaturase (d6FAD) differed. d6FAD is located at the

start of both pathways and adds a double bond to α-linoleic acid (ALA, C18:3n-3) or linoleic acid (LA, C18:2n-6) to form stearidonic acid (SDA, C18:4n-3) or γ-linoleic acid (GLA, C18:3n-6), respectively (Fig. 5.2). There was no significant difference in the expression of d6FAD between growth phases ($F_{2,8}$=3, P=0.0962) or treatments ($F_{1,4}$=3, P=0.1863), even though there was a significant interaction between the two factors ($F_{2,8}$=26, P=0.0003). This is surprising, since SDA, one of the products of d6FAD, is the dominant FA in most phases (Table **5.3**). There are two explanations for this: 1) the Δ^{15}-fatty acid desaturase is actively converting all GLA into SDA; 2) the Δ^{6}-elongase is limiting the production of eicosatetraenoic acid (ETA, C20:4n-3) from SDA, allowing SDA to accumulate despite the low expression levels of d6FAD.

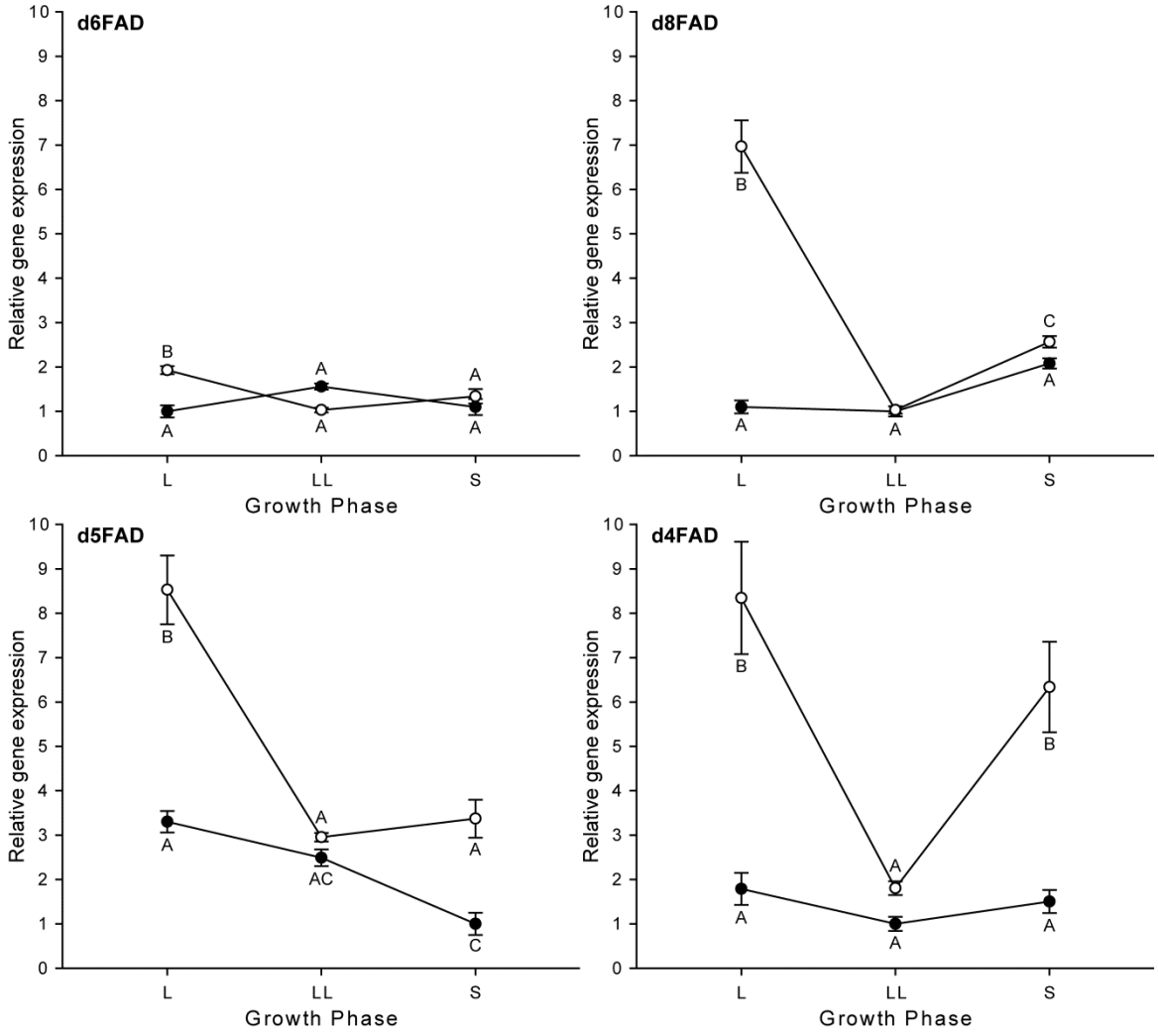

Fig. 5.2 Relative gene expression of Δ^{6}-desaturase (d6FAD), Δ^{8}-desaturase (d8FAD), Δ^{5}-desaturase (d5FAD) and Δ^{4}-desaturase (d4FAD), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), alpha-tubulin (TUA) and beta-tubulin (TUB) as reference genes, in nitrogen-deplete (closed) and nitrogen-replete (open) cultures of *I.* aff. *galbana* (TISO). Mean ± SE is shown, n = 3. Letters indicate significant differences based on Tukey's post-hoc test ($p < 0.05$).

d8FAD is involved in bypassing the d6FAD/Δ^6-elongase step and adds a double bond to either eicosatrienoic acid or eicosadienoic acid to form ETA acid or di-homo-γ-linoleic acid, respectively, while d5FAD adds a double bond to either eicosatetraenoic acid or di-homo-γ-linoleic acid to form eicosapentaenoic acid (EPA) or arachidonic acid (ARA), respectively. Finally, d4FAD adds a double bond to docosapentaenoic acid (C22:5n-3) to form the final product DHA. In contrast to d6FAD, the d8FAD, d5FAD and d4FAD of nitrogen-replete cultures were upregulated 7-, 8.5- and 8.5-fold, respectively, in the logarithmic phase and also in stationary phase 2.5-, 3-, and 6-fold, respectively (Fig. 5.2). The expression of d8FAD and d4FAD in nitrogen-deplete cultures did not vary significantly between growth phases, although, there was a slight non-significant upregulation during the stationary growth phase. Conversely, d5FAD in nitrogen-deplete cultures was downregulated stepwise and was lowest in the stationary growth phase, and also plateaus in the late logarithmic and stationary growth phases of nitrogen-replete cultures.

Statistically, the expression of d8FAD, d5FAD and d4FAD exhibited a significant difference between growth phases (d8FAD: $F_{2,8}$=83, P<0.0001; d5FAD: $F_{2,8}$=51, P<0.0001; d4FAD: $F_{2,8}$=33, P=0.0001) and treatments (d8FAD: $F_{1,4}$=68, P=0.0012; d5FAD: $F_{1,4}$=68, P=0.0012; d4FAD: $F_{1,4}$=24, P=0.0080) with a significant interaction between the two factors (d8FAD: $F_{2,8}$=95, P<0.0001; d5FAD: $F_{2,8}$=18, P=0.0011; d4FAD: $F_{2,8}$=21, P=0.0007). It is interesting to note that none of the intermediate FAs leading to the final product DHA, with the exception DHA itself, were detected at levels > 1 mg g^{-1} AFDW. Conversely, the three corresponding desaturases in the ω-3 pathway (d8FAD, d5FAD and d4FAD) leading to DHA were highly upregulated during the logarithmic growth phase of nitrogen-replete cultures. This could indicate a rapid turnover of these intermediate FAs towards DHA.

At the cellular level, the need of algal cells to maintain adequate levels of DHA during periods of fast growth to maintain membrane integrity, being halved during each cell division, can explain the observed absence of intermediate products and upregulation of the relevant desaturases in nitrogen-replete cultures in logarithmic phase. Curiously, d4FAD, and to a lesser degree d8FAD, were also highly upregulated in the stationary phase of cultures started in nitrogen-replete conditions, while the expression of d5FAD was plateauing in late logarithmic and stationary growth phases of nitrogen-replete conditions. This is in line with the onset of nitrogen starvation and slower biomass growth, as nitrogen was only supplied at the start of the culture period in N+ cultures and would therefore deplete with increased cell growth. This increase in the expression of the three desaturases in stationary phase, most noticeably d4FAD, could be a

response to the onset of nutrient starvation in cultures grown to a high cell density in nitrogen-replete medium, perhaps indicating that they have a higher requirement for DHA for maintaining turnover rates of membrane lipids.

5. Discussion

The FA composition of algae is not constant and varies with changing environmental factors. For example, some microalgae exhibit a shift from a high proportion of PUFAs in logarithmic phase, towards an increased amount of monounsaturated and/or saturated FAs in late logarithmic and stationary phase, where nitrogen becomes limited (Huerlimann et al., 2010). This study in *I.* aff. *galbana* (TISO) also confirms these results but extends these biochemical level results to identifying the molecular drivers (i.e. FA desaturase expression).

The Δ^6-, Δ^8-, Δ^5- and Δ^4-desaturases (d6FAD, d8FAD, d5FAD and d4FAD, respectively) of *I.* aff. *galbana* (TISO) were sequenced for this study, representing all front-end desaturases in the pathway leading to docosahexaenoic acid (DHA, C22:6n-3). The four desaturase sequences were used to investigate the expression of the corresponding genes and resulting impact on the fatty acid (FA) composition during logarithmic, late logarithmic and stationary growth phase in cultures grown in nitrogen-replete and nitrogen-deplete conditions. Results showed that d8FAD, d5FAD and d4FAD are highly upregulated in actively growing nitrogen-replete cultures, while d6FAD is not significantly differentially expressed. Observed changes in expression of the different desaturases corresponded to changes in the FA profile. Upregulation of the three FADs in logarithmic growth phase of nitrogen-replete cultures coincided with rapid cell division and facilitated the supply of the membrane-associated DHA.

Of the intermediate FAs leading to DHA, the accumulation of stearidonic acid (SDA, C18:4n-3) in *I.* aff. *galbana* (TISO) deserves particular attention. Two pathways lead to the formation of SDA: 1) LA can be turned into the ALA by a Δ^{15}-desaturase, which is desaturated into SDA by d6FAD (ω-3 pathway, or 2) LA is turned into GLA by the same d6FAD in the ω-6 pathway, and successively turned into SDA by a Δ^{15}-desaturase. We observed high contents of SDA (19.6 to 34.7 mg g^{-1} AFDW), but LA and ALA were only present at quantities < 10 mg g^{-1} AFDW, and GLA was present at even smaller quantities of ≤ 1.6 mg g^{-1} AFDW. Surprisingly, d6FAD was not significantly differentially expressed with growth phase or nutrient regime in *I.* aff. *galbana* (TISO), which stands in direct conflict to the high content and growth phase-induced slight declines of SDA. The generally high SDA content can be the outcome of different biosynthesis scenarios: 1) the Δ^6-elongase which converts SDA to ecosatetraenoic acid (ETA) may

not be highly expressed and therefore provides a bottleneck for the further biochemical conversion of SDA, or 2) SDA, which is bound to glycerol during that desaturation step, is directed towards a product that is not accessible by the acyl-CoA specific Δ^6-elongase, as has been shown in plants (Abbadi et al., 2004). This partitioning can explain why some algae can accumulate FAs from different parts of the same pathway, for example SDA and DHA, while intermediate FAs occur only at low quantities due to being directly converted along the pathway to the end product.

Another interesting aspect is that, with the exception of LA, ALA and SDA, none of the FA in the ω-3 and ω-6 pathways occurred in quantities above 1.6 mg g^{-1} AFDW. DHA, the final product of the ω-3 pathway, occurs at a constant 11.6 to 12.4 mg g^{-1} AFDW, independent of growth phase or treatment. Conversely, the corresponding FA desaturases were all upregulated when nitrogen is abundant and downregulated in the late logarithmic phase. In algae, DHA has an important function in membrane fluidity (Valentine and Valentine, 2004). It is therefore no surprise that the DHA content is stable between different growth phases. The upregulation of the corresponding desaturases during fast cell growth, as seen during the logarithmic phase in nitrogen-replete cultures, allows the cells to maintain a constant level of DHA. This can also be seen in the upregulation of plastidial acetyl-CoA carboxylase (ACCase), which provides malonyl-CoA for the *de novo* synthesis of FAs as was shown in chapter 4 of this thesis. The upregulation of the expression of d8FAD, d5FAD and d4FAD during active growth and lack of upregulation of d6FAD is in contrast to the expression of a d6FADs and d5FADs in the green freshwater microalgae *Parietochloris incisa* (Iskandarov et al., 2010). Here the upregulation of the d6FADs and d5FADs peaked three days after nutrient starvation. The upregulation of the two desaturases corresponded to the peak production of ARA thus engaging the ω-6 pathway, rather than the ω-3 pathway as found in this study. In view of the results of both studies, it must be concluded that there are inherent differences between different organisms, depending on their ecology, highlighting the importance of investigating a broad range of species from different phyla to elucidate phyla level differences in gene expression as a reaction to environmental conditions. For example, the two closely related strains of *Isochrysis galbana*, TISO and Parke, differ in their lipid composition. While TISO produces DHA, most likely at the expense of building up EPA levels, the Parke strain contains both appreciable amounts of EPA and DHA (Liu and Lin, 2001). It would therefore be worthwhile to investigate different desaturases in both strains and compare their expression with corresponding FA profiles.

The strong upregulation of d4FAD during stationary phase of nutrient-replete cultures is not easily explained. The upregulation did not correlate with larger amounts of DHA, nor with higher productivity due to increased growth rates. One possible explanation is that d4FAD provides another function. However, the cloning and characterisation of a highly similar d4FAD from *Isochrysis galbana* H29 reported a high conversion rate of DPA to DHA with high substrate specificity (Shi et al., 2012), making the existence of an alternative function unlikely. A similar upregulation was observed in stationary phase of nitrogen-replete cultures for plastidial ACCase of *I.* aff. *galbana* (TISO), which also did not correlate with an increase in total FA content as discussed in chapter 4 of this thesis. It is possible that there are distinct differences in *I.* aff. *galbana* (TISO) grown in nitrogen-replete and nitrogen-deplete conditions in the need to maintain membrane integrity in stationary phase either through membrane maintenance due to turnover or, alternatively a higher amount of membranes in older cells of stationary phase cultures. To ascertain that there are indeed different membrane maintenance requirements between cells in late logarithmic and stationary phase grown under different nitrogen regimes, additional investigations combining cell biological, biochemical and molecular tools are required.

In conclusion, this study showed that the expression of fatty acid desaturases in *Isochrysis* aff. *galbana* (TISO) varies with culture age and nitrogen supply, which is reflected in changing levels of FA desaturation. Besides direct knowledge on how nitrogen provision affects FA desaturase gene expression and hence FA profiles, such information can lead to biochemical engineering approaches, which are mandatory for choosing appropriate environmental conditions for improved biomass and FA productivities, in order to achieve an end product-informed desirable FA composition without resorting to genetic engineering. In the case of *Isochrysis* aff. *galbana* (TISO), the content of DHA does not vary significantly between growth phases or nitrogen treatments. Therefore, it is advisable to choose conditions that facilitate fastest growth if the production of DHA is desired. As a well-balanced diet relies on desirable ratios of ω-6 and ω-3 FAs, effects of culture age and culture nutrient status on ω-6/ω-3 ratios should be considered, as a shift in the total amount of ω-3 FAs towards ω-6 FAs was evident. Though not a consideration for *I*. aff. *galbana* (TISO), it is suggested that for applications depending on a low ω-6/ω-3 ratio, cultures might have to be grown in nutrient-sufficient medium and harvested during logarithmic phase. Our results highlight, that future studies should aim to investigate a broader range of taxonomic groups of commercial importance (e.g. Eustigmatophyceae or Bacillariophyceae to name two) and include expression of elongases. In addition, the analysis of the FA

composition of the different forms of FA pools (i.e. triacylglycerols, phospholipids, acyl-CoA, free fatty acids, etc.) is advisable in order to unravel the occurrence of sinks, removing specific FAs from the elongation/desaturation pathway and allowing their accumulation.

Chapter 6 - General discussion

As outlined in chapter 1 and throughout this thesis, algae have a vast as valuable, sustainable resources for products such as biodiesel for transportation, ω-3 fatty acids (FAs) for human consumption and bio-polymers with a wide variety of applications. However, the full potential of algae has not yet been fully exploited. To improve yields of desirable target FAs, new approaches should integrate phylogenetic data and molecular analysis (at least at the gene expression level) of key enzymes in the lipid synthesis pathway. Prior to the commencement of the present research, there were several gaps in the current knowledge on algal fatty acid synthesis, and in particular the key enzyme acetyl-CoA carboxylase (ACCase). The fatty acid (FA) synthesis pathway was thought to be closely related to plants, but information was scattered. Furthermore, heteromeric ACCase was generally cited as being present in the plastids of algae (e.g. Guarnieri et al. (2013)), while homomeric ACCase was rarely considered. Lastly, while there has been decades of research on algal lipid content and composition under different environmental conditions (e.g. Martinez-Fernandez et al. (2006), Converti et al. (2009), Liang et al. (2009), Rodolfi et al. (2009) and Yoo et al. (2010)), there was a distinct lack of data on the expression of genes involved in lipid *de novo* synthesis and desaturation.

To address these knowledge gaps, a comprehensive series of studies were undertaken that summarised the lipid synthesis pathway, investigated the presence of heteromeric and homomeric ACCase in the plastids of algae, analysed the phylogeny of ACCase and measured the gene expression of homomeric ACCase and selected desaturases. This research has contributed towards the understanding of the lipid synthesis pathways in algae, which provides the groundwork for improving FA yields in algal lipid production.

The following discussion first summarises the major conclusions and outcomes obtained in the previous chapters. Of particular interest is the finding that all investigated algae with a plastid of secondary or tertiary endosymbiotic origin, which makes up the majority of the algal diversity, contain homomeric ACCase in their plastids. The summary of the major conclusions and outcomes is followed by a synthesis of the results and a discussion of the potential applications of the present research. Finally, the discussion concludes by addressing future research directions.

1. Major conclusions and outcomes

A review of the lipid synthesis pathway in algae opened chapter 2, followed by an analysis of heteromeric and homomeric ACCase sequences in relation to taxonomic classification of algae. The first major contribution of this chapter is that the presence of heteromeric or homomeric ACCase is dependent on the origin of the plastid. Heteromeric ACCase was found in plastids of most green algae and all red algae. These plastids are of primary symbiotic origin and are surrounded by two envelope membranes. The only exception consists of the green algal class Prasinophyceae, which contain homomeric ACCase in their plastids and in which none of the subunits of heteromeric ACCase were found. In contrast, homomeric ACCase was found in all investigated algae with a plastid derived through secondary endosymbiosis. This included the Chlorarachniophyta, Cryptophyta, Stramenopiles and Haptophyta, as well as the apicoplast-containing Apicomplexa. This shows that the majority of the algal taxa contain homomeric ACCase in their plastids.

The second major contribution of this chapter not only allows distinguishing between heteromeric and homomeric ACCase, but also between the plastidial and cytosolic localisation of homomeric ACCase. For this, the amino acid sequences of the substrate binding regions of the biotin carboxylase, α-carboxyltransferase (α-CT) and β-carboxyltransferase subunits/domains were analysed, revealing distinct differences between heteromeric and homomeric ACCase. This allows an identification of the ACCase type based on these binding regions alone. Furthermore, the first three amino acids of the acetyl-CoA binding region of homomeric α-CT include a distinct motif that differs between cytosolic and plastidial localisations of homomeric ACCase. These distinct motifs were used to identify the localisation of the ACCase genes that have been sequenced in chapter 3.

Chapter 3 investigated the phylogeny of plastidial homomeric and heteromeric ACCase and compared it to differing views on algal taxonomy. Cytosolic ACCase is found in all eukaryotes and provides information on the phylogenetic relationships between the eukaryotic host cells. Conversely, the plastidial ACCase of algae with a secondary/tertiary endosymbiotic plastid could originate from a gene duplication of the cytosolic ACCase, from the endosymbiont, or from a horizontal gene transfer. A major finding of this analysis is that the phylogenies of cytosolic and plastidial ACCase support the inclusion of the Rhizaria with the Stramenopiles and Alveolata as SAR. This is consistent with the findings of other genetic (Pawlowski, 2013) and ultra-structural studies (Yubuki and Leander, 2013). Considering the Chlorarachniophytes

(Rhizaria) contain a plastid derived from a green alga (Rogers et al., 2007) and the Stramenopiles and Alveolata contain a plastid derived from a red alga (Archibald, 2009b), the close relationship of the SAR host species could be explained by a common heterotrophic ancestor, which diverged into the two lines as a red and a green alga were taken up, respectively. However, this would not explain the close relationship between the plastidial ACCase of the SAR, Cryptophyta, Haptophyta and the green Prasinophyceae. The presence of an ancestral cryptic plastid of green origin in the red lineage has been largely refuted (Archibald, 2009a, Deschamps and Moreira, 2012). This conundrum can be explained by a serial secondary endosymbiotic event in the Chlorarachniophyta (Archibald, 2012, Archibald, 2009a). Here, a red plastid containing ancestor of the SAR, Haptophyta, and Cryptophyta, acquired the plastidial ACCase gene from an ancestor or relative of the Prasinophyceae. This was followed by a serial loss of the red plastid in the Rhizaria, and the acquisition of a green plastid in the Chlorarachniophyta.

Finally, the phylogeny of the plastid encoded β-carboxyltransferase was also analysed. The most interesting finding is the strong support for a clade made up of cyanobacteria and the Rhizaria *Paulinella chromatophora*. This strong statistical support provides additional evidence for a second independent and more recent primary endosymbiotic event (Marin et al., 2005, Nakayama and Ishida, 2009). Until recently, the primary endosymbiosis of a cyanobacterium was considered to only have occurred once, leading to the primary endosymbiotic plastid containing land plants, glaucocystophytes and red and green algae. The fact that all four subunits of the heteromeric ACCase are found in the chromatophore (plastid) genome of *P. chromatophora* (Nowack et al., 2008), while the plastid genome of all other heteromeric ACCase containing photosynthetic eukaryotes only encodes for the β-CT subunit further supports this hypothesis.

Chapter 4 investigated the expression of the ACCase transcripts of *I.* aff. *galbana* (TISO) and *Chromera velia* that were sequenced in chapter 3. The major finding of this chapter is that the expression of plastidial ACCase correlates strongly with FA production. In *C. velia*, the expression of ACCase and the FA content increased with culture age and were significantly higher in nitrogen limited cultures. In contrast, *I.* aff. *galbana* (TISO) showed no significant differences in FA content between different growth phases and nutrient availability. However, the plastidial ACCase was highly upregulated during the logarithmic growth phase of nitrogen-replete cultures. This explained the steady FA content in actively dividing cells, compared to cells in stationary phase. ACCase is highly upregulated to provide the dividing cells with

adequate supplies of FA to build membranes during cell division. This demonstrates that there are distinct differences in the expression of genes involved in lipid synthesi between algal taxa.

Chapter 5 investigated the expression of the four front-end desaturases involved in the production of docosahexaenoic acid (DHA, C22:6n-3) in of *I.* aff. *galbana* (TISO). The major finding of this chapter is that the main desaturases involved in DHA synthesis (Δ8-, Δ5- and Δ4-desaturase) are highly upregulated during the logarithmic phase of nitrogen-replete cultures. DHA plays an important role in the membranes of algae (Guschina and Harwood, 2006, Murata and Siegenthaler, 1998). This explains the higher expression of the desaturases to maintain adequate levels of DHA during rapid cell division. Furthermore, the DHA content does not change significantly between different growth phases and nutrient treatments, however, there is a significant shift in the ω-3/ω-6 ratio towards ω-6 FAs in nutrient-deplete conditions or later growth phases.

2. Synthesis and applications of research outcomes

2.1 Lipid metabolism in algae

The lipid metabolism in algae is a complex system, consisting of the *de novo* synthesis, elongation and desaturation of fatty acids (FAs), the synthesis of neutral and polar lipids, the mobilization of FAs from lipids, and the β-oxidation of FA. The present research focused on the *de novo* synthesis, elongation and desaturation of FAs. The pathway outlined in chapter 2 of this thesis represents a generalised core pathway, based on information from plants and other model organisms. However, lipid metabolism in algae is still poorly understood and the actual pathways may deviate from this generalization (Obata et al., 2013). For example, while triacylglycerides (TAGs) are generally thought to be synthesised in the smooth endoplasmic reticulum, it has been shown that *Chlamydomonas reinhardtii* exhibits a TAG pathway in its chloroplast (Fan et al., 2011), which is possibly also present in other algae. As more transcriptome, proteome and metabolome studies are conducted and more enzyme kinetics data are made available, our picture of the lipid metabolism in algae will become clearer (Obata et al., 2013). The differences in the expression of plastidial ACCase and resulting differences FA content of *I.* aff. *galbana* (TISO) and *Chromera velia* presented in chapter 4 of this thesis can most likely also be found in other enzymes involved in the lipid metabolism, for example the lysophosphatidic acid- and diacylglycerol acyltransferases, which determine which fatty acids are incorporated into TAGs and phospholipids. These differences in expression and specificity can explain

part of the astounding variation in fatty acid composition found in algae. It is furthermore important to keep in mind that the grouping "algae" encompasses a large number of phyla, having arisen from several endosymbiotic events, rampant theft of genes (i.e. horizontal gene transfer) and repurposing of genes for other pathways, as touched upon in chapter 2 and 3, resulting in a large genetic variation. It is likely that there is not only divergence in how the lipid metabolism is regulated in different algae, but also divergence in the actual pathways involved.

While evolutionarily advantageous, this genetic variation, along with the repurposing of genes in different pathways and general complexity, increases the difficulty to untangle the complicated evolutionary history of algae. For example, the presence of ACCase in the plastids and cytosol of algae makes it difficult to identify the localisation of the enzyme, making it important to find ways to differentiate between two transcripts.

2.2 Identification of cytosolic and plastidial homomeric ACCase

In most algae containing a plastid derived from a primary endosymbiotic event, the differentiation of cytosolic and plastidial ACCase is easier due to the respective presence of the homomeric and heteromeric forms. In contrast, in algae with plastids derived from a secondary/tertiary endosymbiotic event, and in the green algal class Prasinophyceae, the presence of homomeric ACCase in the cytosol as well as the plastids, makes expression studies more difficult. Several studies report on the expression of homomeric ACCase (Cheng et al., 2013, Liang et al., 2013); however, since the localisation of their ACCase transcript is not identified, it cannot be certain whether plastidial or cytosolic ACCase was measured. In chapter 2, I proposed the use of the amino acid motif GKS in the acetyl-CoA binding region of the α-carboxyltransferase domain to identify plastidial ACCase. The only variation found so far is *Chromera velia*, as outlined in chapter 3, which contains the motive GKG. Regardless, the motif will be useful in transcriptome studies to distinguish between the plastidial and cytosolic form. However, limitations of this approach will be discussed in the section "Research recommendations" below.

2.3 Use of phylogenetic and metabolic information to improve lipid yields

Be it FAs for biodiesel or polymer production, or specific very long-chain polyunsaturated fatty acids (VLC-PUFAs) for human consumption, increasing productivity of target FAs is an important step to making a product economically and ecologically viable. Next to approaches dealing with the lipid synthesis pathway investigated in the present thesis, other parts of lipid metabolism can also be targeted.

For example, instead of targeting the synthesis of FAs, Trentacoste et al. (2013) knocked out a lipase in the diatom *Thalassiosira pseudonana,* which reduces the amount of FAs that are freed from storage lipids to be used in β-oxidation, resulting in the accumulation of FAs. In contrast, Li et al. (2010) produced a starchless mutant of *Chlamydomonas reinhardtii*, resulting in the hyper-accumulation of TAGs, since energy and carbon was diverted away from starch accumulation. Extensive knowledge of the complex lipid metabolism, as discussed above, provides the opportunity to use biochemical engineering and molecular engineering to improve yields. Biochemical engineering here refers to improving lipid production through environmental factors (e.g. nutrients, temperature, pH or salinity), while molecular engineering can be used to increase productivity through overexpression of certain genes, but also designing new pathways foreign to the organism (Courchesne et al., 2009).

A good general example for a biochemical engineering approach and the importance of phylogeny are the differences in ACCase expression presented in chapter 4, and references therein. As described, some algae accumulate FAs during nitrogen starvation, while others only exhibit a shift from VLC-PUFAs to short-chain FAs with no change in total FA content. This metabolic change is also reflected at a genetic level in the differences in gene expression of ACCase. However, different algal species will react differently to biochemical approaches, making it important to combine the metabolic knowledge with phylogeny. In practice, if the aim was for example to produce a bio-based polymer from algal oleic acid (C18:1n-9), *Isochrysis* aff. *galbana* (TISO) could be grown under nitrogen-limited conditions to stationary phase, based on the finding in chapter 5. While the resulting ash-free dry weight is higher in cultures grown under nitrogen-replete conditions, the increase in oleic acid in cultures grown in nitrogen-deplete conditions more than makes up for this.

In terms of molecular engineering, it is important to identify and alleviate bottlenecks in the native pathways. However, alleviating one bottleneck might lead to bottlenecks in other parts of the pathway. For example, the algal desaturation and elongation pathway has been recreated in plants, without much success. One possible bottleneck in this process could be the availability of malonyl-CoA, produced by cytosolic ACCase. As it has been shown in chapter 4 of this thesis, there is a strong correlation between cytosolic ACCase and the length of fatty acids. In practice, this could mean that when genetically modifying an organism with, for example a designed fatty acid desaturation and elongation pathway to increase the production of long-chain polyunsaturated fatty acids, it could be required to increase the expression of ACCase in this organism to supply malonyl-CoA for the elongation process.

Combining the biochemical and molecular engineering approaches allows for even greater flexibility. For example, a genetically modified alga which contains an acyltransferase with preference of including VLC-PUFAs into TAG and whose lipase has been knocked out could be grown in nutrient replete conditions to hypothetically accumulate VLC-PUFAs in TAG storage bodies. Alternatively, if short-chain saturated and monounsaturated FAs are desired for the production of biodiesel, interfering with the expression of cytosolic ACCase could lead to shorter, saturated FAs, while still maintaining high growth rates under nutrient-replete conditions.

In summary, the combination of different methods to manipulate the lipid productivity and composition through biochemical and/or molecular engineering, based on our knowledge of the different pathways of lipid metabolism in algae, their connection and regulation, as well as species specific differences, will result in increased lipid productivity.

3. Research recommendations

During this research, and as highlighted throughout the thesis, a number of areas were identified that require further investigation. These will briefly be summarised here.

3.1 Algal ACCase phylogeny and identification of localisation

It was proposed that all algae with a secondary/tertiary plastid contain homomeric ACCase. However, ACCase sequences of the Euglenophyta, Glaucocystophyta and Dinophyta are still missing. As additional genomes are being sequenced and become publicly available, it will be possible to draw a more complete picture, and also provide better resolution for algal phylogeny. Considering all the investigated plastidial ACCases of algae are phylogenetically related, more sequences of different strains of Prasinophyceae and other Chlorophyta species would provide answers to some of the open questions of the origins of plastidial homomeric ACCase and algal phylogeny in general. Since the Euglenophyta contain a plastid of secondary endosymbiotic origin, while the host is not closely related to the SAR, Haptophyta and Cryptophyta, it would be especially interesting to see which type of ACCase the contain in their plastid and where this ACCase fits in with the other taxa phylogenetically. Lastly, even though the proposed motif to identify the plastidial homomeric ACCase is based on clearly identified sequences and has so far held true for all investigated algae, it has not been experimentally proven. Furthermore, chapter 3 showed that the investigated plastidial ACCase are all phylogenetically related. It would therefore be interesting to investigate algae that are not as closely phylogenetically related, for example the Euglenophyta.

3.2 Expression of ACCase and desaturases correlated with fatty acid productivity and composition

The present study only investigated total lipid and FA content. The compartmentalisation of FAs into different lipid types seems to be an important mechanism to regulate FAs composition in algae, most likely because the chemical properties of certain types of FAs are suitable for different functions in the cell. Further research should therefore investigate the content and FA composition of the different lipid types (e.g. TAG, phospholipids, galactolipids) separately.

Furthermore, even though qPCR is relatively inexpensive, sensitive, and does not require extensive bioinformatics steps, complete transcriptome analyses are becoming more and more common due to the rapid decrease in the cost of next-generation sequencing and the availability of complete analysis pipelines. Transcriptome studies will allow for further advances in our knowledge of the lipid metabolism in algae, especially when coupled with broader metabolic data as mentioned above. These studies should include a broad range of taxa and stress conditions.

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Appendix

Supplemental Table 1 Complete list of heteromeric ACCase subunits and homomeric ACCase with corresponding identification numbers and sources.

Species	Taxonomy	Protein	Identification	Source
Toxoplasma gondii	Apicomplexa	ACC1	AAF04493	NCBI
Toxoplasma gondii	Apicomplexa	ACC2	AAF04494	NCBI
Toxoplasma gondii (GT1)	Apicomplexa	ACCase	EEE20751	NCBI
Toxoplasma gondii (ME49)	Apicomplexa	ACC1	XP_002369961	NCBI
Toxoplasma gondii (ME49)	Apicomplexa	ACC2	XP_002370640	NCBI
Toxoplasma gondii (VEG)	Apicomplexa	ACC1	EEE30125	NCBI
Toxoplasma gondii (VEG)	Apicomplexa	ACC2	EEE28725	NCBI
Cyclotella cryptica	Bacillariophyceae	ACCase	AAA81471	NCBI
Fragilariopsis cylindrus (CCMP1102)	Bacillariophyceae	ACCase	274583	JGI
Fragilariopsis cylindrus (CCMP1102)	Bacillariophyceae	ACCase	145710	JGI
Phaeodactylum tricornutum	Bacillariophyceae	ACCase	54926	JGI
Phaeodactylum tricornutum	Bacillariophyceae	ACCase	55209	JGI
Thalassiosira pseudonana	Bacillariophyceae	ACCase	6770	JGI
Thalassiosira pseudonana	Bacillariophyceae	ACCase	12234	JGI
Escherichia coli (K12)	Bacteria	α-CT	P0ABD5	NCBI
Escherichia coli (K12)	Bacteria	BC	P24182	NCBI
Escherichia coli (K12)	Bacteria	BCCP	P0ABD8	NCBI
Escherichia coli (K12)	Bacteria	β-CT	P0A9Q5	NCBI
Arabidopsis thaliana	Brassicaceae	ACC1	NP_174849	NCBI
Arabidopsis thaliana	Brassicaceae	ACC2	NP_174850	NCBI
Arabidopsis thaliana	Brassicaceae	α-CT	Q9LD43	NCBI
Arabidopsis thaliana	Brassicaceae	BC	O04983	NCBI
Arabidopsis thaliana	Brassicaceae	BCCP1	Q42533	NCBI
Arabidopsis thaliana	Brassicaceae	BCCP2	Q9LLC1	NCBI
Arabidopsis thaliana	Brassicaceae	β-CT	P56765	NCBI
Physcomitrella patens patens	Bryophyte	ACCase	EDQ80874	NCBI
Physcomitrella patens patens	Bryophyte	ACCase	EDQ62113	NCBI
Bigelowiella natans	Chlorarachniophyta	ACC1	92488	JGI
Bigelowiella natans	Chlorarachniophyta	ACC2	18464	JGI
Auxenochlorella protothecoides	Chlorophyta	β-CT	AEU08409	NCBI
Bathycoccus prasinos	Chlorophyta	ACCase	CCO17834	NCBI
Bathycoccus prasinos	Chlorophyta	ACCase	CCO18633	NCBI
Bryopsis hypnoides	Chlorophyta	β-CT	ACX33787	NCBI
Chara vulgaris	Chlorophyta	β-CT	ABA61965	NCBI
Chlorococcum humicola	Chlorophyta	β-CT	ADZ15145.1	NCBI
Chlamydomonas reinhardtii	Chlorophyta	α-CT	184945	JGI
Chlamydomonas reinhardtii	Chlorophyta	BC	122970	JGI
Chlamydomonas reinhardtii	Chlorophyta	BCCP1	185478	JGI
Chlamydomonas reinhardtii	Chlorophyta	BCCP2	183660	JGI
Chlamydomonas reinhardtii	Chlorophyta	β-CT	133238	JGI
Chlorella sp. (NC64A)	Chlorophyta	ACCase	56737	JGI
Chlorella sp. (NC64A)	Chlorophyta	α-CT	36222	JGI
Chlorella sp. (NC64A)	Chlorophyta	BC	140567	JGI
Chlorella sp. (NC64A)	Chlorophyta	BCCP1	142410	JGI
Chlorella sp. (NC64A)	Chlorophyta	BCCP2	136966	JGI
Chlorella variabilis	Chlorophyta	β-CT	ADZ04992	NCBI
Chlorella vulgaris	Chlorophyta	β-CT	BAA57908	NCBI

Species	Taxonomy	Protein	Identification	Source
Coccomyxa subellipsoidea (C-169)	Chlorophyta	α-CT	EIE26011	NCBI
Coccomyxa subellipsoidea (C-169)	Chlorophyta	BC	EIE19193	NCBI
Coccomyxa subellipsoidea (C-169)	Chlorophyta	BCCP	EIE25224	NCBI
Coccomyxa subellipsoidea (C-169)	Chlorophyta	β-CT	ADV29875	NCBI
Dunaliella salina	Chlorophyta	β-CT	ABO33321	NCBI
Haematococcus pluvialis	Chlorophyta	BC	ABS45106.1	NCBI
Helicosporidium sp.	Chlorophyta	β-CT	ABD33968	NCBI
Leptosira terrestris	Chlorophyta	β-CT	ABO69279	NCBI
Micromonas pusilla (CCMP1545)	Chlorophyta	ACCase	31755	JGI
Micromonas pusilla (CCMP1545)	Chlorophyta	ACCase	16401	JGI
Micromonas sp. (RCC2999)	Chlorophyta	ACCase	113382	JGI
Micromonas sp. (RCC2999)	Chlorophyta	ACCase	104872	JGI
Muriella zofingiensis	Chlorophyta	BC	ACX71633.1	NCBI
Nephroselmis olivacea	Chlorophyta	β-CT	AF137379_28	NCBI
Oltmannsiellopsis viridis	Chlorophyta	β-CT	ABB81983	NCBI
Oocystis solitaria	Chlorophyta	β-CT	ACQ90742	NCBI
Ostreococcus lucimarinus	Chlorophyta	ACCase	44400	JGI
Ostreococcus lucimarinus	Chlorophyta	ACCase	11	JGI
Ostreococcus sp. (RCC809)	Chlorophyta	ACCase	61393	JGI
Ostreococcus sp. (RCC809)	Chlorophyta	ACCase	36970	JGI
Ostreococcus tauri	Chlorophyta	ACCase	CAL50235	NCBI
Ostreococcus tauri	Chlorophyta	ACCase	CAL56314	NCBI
Parachlorella kessleri	Chlorophyta	β-CT	ACQ90907	NCBI
Pedinomonas minor	Chlorophyta	β-CT	ACQ90825	NCBI
Polytomella parva	Chlorophyta	BCCP	ABH11004	NCBI
Pseudendoclonium akinetum	Chlorophyta	β-CT	AAV80676	NCBI
Trebouxia aggregata	Chlorophyta	β-CT	ABX82657	NCBI
Trebouxiophyceae sp.	Chlorophyta	β-CT	AFQ93852	NCBI
Volvox carteri f. *nagariensis*	Chlorophyta	ACCase	106840	JGI
Volvox carteri f. *nagariensis*	Chlorophyta	α-CT	120546	JGI
Volvox carteri f. *nagariensis*	Chlorophyta	BC	77540	JGI
Volvox carteri f. *nagariensis*	Chlorophyta	BCC1	81953	JGI
Volvox carteri f. *nagariensis*	Chlorophyta	BCC2	109910	JGI
Volvox carteri f. *nagariensis*	Chlorophyta	β-CT	82311	JGI
Guillardia theta	Cryptophyta	ACC1	68771	JGI
Guillardia theta	Cryptophyta	ACC2	72035	JGI
Anabaena variabilis (ATCC 29413)	Cyanobacteria	α-CT	Q3MA36	NCBI
Anabaena variabilis (ATCC 29413)	Cyanobacteria	BC	YP_321035	NCBI
Anabaena variabilis (ATCC 29413)	Cyanobacteria	BCCP	YP_322827	NCBI
Anabaena variabilis (ATCC 29413)	Cyanobacteria	β-CT	YP_320706	NCBI
Microcystis aeruginosa NIES-843	Cyanobacteria	α-CT	BAG00629	NCBI
Microcystis aeruginosa NIES-843	Cyanobacteria	BC	BAG01493	NCBI
Microcystis aeruginosa NIES-843	Cyanobacteria	BCCP	BAG05514	NCBI
Microcystis aeruginosa NIES-843	Cyanobacteria	β-CT	BAG05575	NCBI
Nostoc azollae (0708)	Cyanobacteria	α-CT	ZP_03763675	NCBI
Nostoc azollae (0708)	Cyanobacteria	BC	ZP_03762854	NCBI
Nostoc azollae (0708)	Cyanobacteria	BCCP	ZP_03766533	NCBI
Nostoc azollae (0708)	Cyanobacteria	β-CT	ZP_03766652	NCBI
Nannochloroposis gaditana	Eustigmatophycea	ACC1	AFJ69228	NCBI
Emiliana huxleyi (1516)	Haptophyta	ACCase	455280	JGI
Emiliana huxleyi (1516)	Haptophyta	ACCase	449545	JGI
Phytophthora infestans (T30-4)	Stramenopiles	ACC	EEY68805	NCBI
Phytophthora sojae	Stramenopiles	ACC	EGZ09435	NCBI

Species	Taxonomy	Protein	Identification	Source
Huperzia lucidula	Lycopodiophyta	β-CT	AAT80716	NCBI
Aureococcus anophagefferens (1984)	Pelagophyceae	ACCase	38836	JGI
Aureococcus anophagefferens (1984)	Pelagophyceae	ACCase	65524	JGI
Ectocarpus siliculosus	Phaeophyta	ACCase	Esi0198_0003	BOGAS
Ectocarpus siliculosus	Phaeophyta	ACCase	Esi0592_0001	BOGAS
Ectocarpus siliculosus	Phaeophyta	ACCase	Esi0367_0023	BOGAS
Ectocarpus siliculosus	Phaeophyta	ACCase	Esi0403_0027	BOGAS
Aegilops tauschii	Poaceae	ACC2	ACD46664	NCBI
Aegilops tauschii	Poaceae	ACC1	ACD46679	NCBI
Triticum urartu	Poaceae	ACC2	ACD46670	NCBI
Triticum urartu	Poaceae	ACC1	ACD46677	NCBI
Paulinella chromatophora	Rhizaria	α-CT	ACB43305	NCBI
Paulinella chromatophora	Rhizaria	BC	ACB43092	NCBI
Paulinella chromatophora	Rhizaria	BCCP	ACB42862	NCBI
Paulinella chromatophora	Rhizaria	β-CT	ACB42560	NCBI
Antithamnion sp.	Rhodophyta	α-CT	P46316	NCBI
Cyanidium caldarium	Rhodophyta	α-CT	O19903	NCBI
Cyanidium caldarium	Rhodophyta	BCCP	O19918	NCBI
Cyanidium caldarium	Rhodophyta	β-CT	Q9TLW3	NCBI
Cyanidioschyzon merolae	Rhodophyta	ACCase	CMM188C	CMGP
Cyanidioschyzon merolae	Rhodophyta	α-CT	CMV056C	CMGP
Cyanidioschyzon merolae	Rhodophyta	BC	CMS299C	CMGP
Cyanidioschyzon merolae	Rhodophyta	BCCP	CMV134C	CMGP
Cyanidioschyzon merolae	Rhodophyta	β-CT	CMV207C	CMGP
Galdieria sulphuraria	Rhodophyta	ACC2	EME32530	NCBI
Galdieria sulphuraria	Rhodophyta	α-CT	EME28461	NCBI
Galdieria sulphuraria	Rhodophyta	BC Isoform 1	EME31879	NCBI
Galdieria sulphuraria	Rhodophyta	BC Isoform 2	EME31878	NCBI
Galdieria sulphuraria	Rhodophyta	BCCP	EME32228	NCBI
Galdieria sulphuraria	Rhodophyta	β-CT	EME28527	NCBI
Gracilaria tenuistipitata var. liui	Rhodophyta	α-CT	AAT79762	NCBI
Gracilaria tenuistipitata var. liui	Rhodophyta	BCCP	AAT79690	NCBI
Gracilaria tenuistipitata var. liui	Rhodophyta	β-CT	AAT79592	NCBI
Porphyra purpurea	Rhodophyta	α-CT	P51371	NCBI
Porphyra purpurea	Rhodophyta	BCCP	P51283	NCBI
Porphyra purpurea	Rhodophyta	β-CT	P51198	NCBI
Porphyra umbilicalis	Rhodophyta	α-CT	AFC40039	NCBI
Porphyra umbilicalis	Rhodophyta	BCCP	AFC39952	NCBI
Porphyra umbilicalis	Rhodophyta	β-CT	AFC39868	NCBI
Porphyra yezoensis	Rhodophyta	α-CT	Q1XDB6	NCBI
Porphyra yezoensis	Rhodophyta	BCCP	Q1XDK5	NCBI
Porphyra yezoensis	Rhodophyta	β-CT	Q1XDT9	NCBI
Chaetosphaeridium globosum	Streptophyta	β-CT	AAM96498	NCBI
Chlorokybus atmophyticus	Streptophyta	β-CT	ABM87949	NCBI
Staurastrum punctulatum	Streptophyta	β-CT	AAX45681	NCBI
Zygnema circumcarinatum	Streptophyta	β-CT	AAX45794	NCBI

Supplemental Table 2 Enzyme designations and NCBI ID numbers of the species used for the BLAST search of the full algal genomes.

Species	Genes	NCBI ID
Chlaydomonas reinhardtii	BC	XP_001702319
Chlaydomonas reinhardtii	BCC1	XP_001700442
Chlaydomonas reinhardtii	BCC2	XP_001690119
Chlaydomonas reinhardtii	α-CT	XP_001696945
Chlaydomonas reinhardtii	β-CT	XP_001703187
Arabidopsis thaliana	ACC1	Q38971
Arabidopsis thaliana	BC	004983
Arabidopsis thaliana	BCCP1	Q452533
Arabidopsis thaliana	BCCP2	Q9LLC1
Arabidopsis thaliana	α-CT	Q9LD43
Arabidopsis thaliana	β -CT	P56765
Cyclotella cryptica	ACC1	AAA81471
Escherichia coli	BC	P24182
Escherichia coli	BCCP	P0ABD8
Escherichia coli	α-CT	P0ABD5
Escherichia coli	β -CT	P0A9Q5

Supplemental Table 3 Species names, taxonomy, protein identification, size, identification numbers and sources of sequences used for the phylogenetic analysis of homomeric ACCase.

Species	Taxonomy	Protein	Size	Identification	Source
Toxoplasma gondii	Apicomplexa	ACC1	2564	AAF04493	NCBI
Toxoplasma gondii	Apicomplexa	ACC2	1102	AAF04494	NCBI
Toxoplasma gondii (GT1)	Apicomplexa	ACCase	2252	EEE20751	NCBI
Toxoplasma gondii (ME49)	Apicomplexa	ACC1	2599	XP_002369961	NCBI
Toxoplasma gondii (ME49)	Apicomplexa	ACC2	3399	XP_002370640	NCBI
Toxoplasma gondii (VEG)	Apicomplexa	ACC1	2612	EEE30125	NCBI
Toxoplasma gondii (VEG)	Apicomplexa	ACC2	3373	EEE28725	NCBI
Cyclotella cryptica	Bacillariophyceae	ACCase	2089	AAA81471	NCBI
Fragilariopsis cylindrus (CCMP1102)	Bacillariophyceae	ACCase	2045	274583	JGI
Fragilariopsis cylindrus (CCMP1102)	Bacillariophyceae	ACCase	2302	145710	JGI
Phaeodactylum tricornutum	Bacillariophyceae	ACCase	2093	54926	JGI
Phaeodactylum tricornutum	Bacillariophyceae	ACCase	2283	55209	JGI
Thalassiosira pseudonana	Bacillariophyceae	ACCase	2089	6770	JGI
Thalassiosira pseudonana	Bacillariophyceae	ACCase	2251	12234	JGI
Arabidopsis thaliana	Brassicaceae	ACC1	2254	NP_174849	NCBI
Arabidopsis thaliana	Brassicaceae	ACC2	2356	NP_174850	NCBI
Physcomitrella patens patens	Bryophyte	ACCase	2238	EDQ80874	NCBI
Physcomitrella patens patens	Bryophyte	ACCase	2249	EDQ62113	NCBI
Bigelowiella natans	Chlorarachniophyta	ACC1	2031	92488	JGI
Bigelowiella natans	Chlorarachniophyta	ACC2	2053	18464	JGI
Bathycoccus prasinos	Chlorophyta	ACCase	2120	CCO17834	NCBI
Bathycoccus prasinos	Chlorophyta	ACCase	2177	CCO18633	NCBI
Chlorella sp. (NC64A)	Chlorophyta	ACCase	1868	56737	JGI
Micromonas pusilla (CCMP1545)	Chlorophyta	ACCase	2146	31755	JGI
Micromonas pusilla (CCMP1545)	Chlorophyta	ACCase	2094	16401	JGI
Micromonas sp. (RCC2999)	Chlorophyta	ACCase	2140	113382	JGI
Micromonas sp. (RCC2999)	Chlorophyta	ACCase	2190	104872	JGI
Ostreococcus lucimarinus	Chlorophyta	ACCase	2013	44400	JGI
Ostreococcus lucimarinus	Chlorophyta	ACCase	1994	11	JGI
Ostreococcus sp. (RCC809)	Chlorophyta	ACCase	2014	61393	JGI
Ostreococcus sp. (RCC809)	Chlorophyta	ACCase	2060	36970	JGI
Ostreococcus tauri	Chlorophyta	ACCase	2123	CAL50235	NCBI
Ostreococcus tauri	Chlorophyta	ACCase	1983	CAL56314	NCBI
Volvox carteri f. *nagariensis*	Chlorophyta	ACCase	2462	106840	JGI
Chromera velia	Chromerida	ACC1	2082	KF673098	Thesis
Chromera velia	Chromerida	ACC2	-	KF673095-7	Thesis
Guillardia theta	Cryptophyta	ACC1	2055	68771	JGI
Guillardia theta	Cryptophyta	ACC2	2171	72035	JGI
Nannochloroposis gaditana	Eustigmatophycea	ACC1	2139	AFJ69228	NCBI
Nannochloroposis oculata	Eustigmatophycea	ACC1	2,317	KF673100	Thesis
Nannochloroposis oculata	Eustigmatophycea	ACC2	2,362	KF673101	Thesis
Emiliana huxleyi (1516)	Haptophyta	ACCase	1577	455280	JGI
Emiliana huxleyi (1516)	Haptophyta	ACCase	2013	449545	JGI
Isochrysis aff. *galbana* (TISO)	Haptophyta	ACC1	2134	KF673099	Thesis
Phytophthora infestans (T30-4)	Stramenopiles	ACC	2326	EEY68805	NCBI
Phytophthora sojae	Stramenopiles	ACC	2310	EGZ09435	NCBI
Aureococcus anophagefferens (1984)	Pelagophyceae	ACCase	1809	38836	JGI
Aureococcus anophagefferens (1984)	Pelagophyceae	ACCase	2153	65524	JGI
Ectocarpus siliculosus	Phaeophyta	ACCase	1848	Esi0198_0003	BOGAS
Ectocarpus siliculosus	Phaeophyta	ACCase	799	Esi0592_0001	BOGAS
Ectocarpus siliculosus	Phaeophyta	ACCase	1049	Esi0367_0023	BOGAS

Species	Taxonomy	Protein	Size	Identification	Source
Ectocarpus siliculosus	Phaeophyta	ACCase	166	Esi0403_0027	BOGAS
Aegilops tauschii	Poaceae	ACC2	2258	ACD46664	NCBI
Aegilops tauschii	Poaceae	ACC1	2311	ACD46679	NCBI
Triticum urartu	Poaceae	ACC2	2260	ACD46670	NCBI
Triticum urartu	Poaceae	ACC1	2311	ACD46677	NCBI
Cyanidioschyzon merolae	Rhodophyta	ACCase	2719	CMM188C	CMGP
Galdieria sulphuraria	Rhodophyta	ACC2	2482	EME32530	NCBI

Supplemental Table 4 Species names, taxonomy, protein identification, size, identification numbers and sources of sequences used for the phylogenetic analysis of the β-carboxyltransferase subunit of heteromeric ACCase.

Species	Taxonomy	Protein	Size	Identification	Source
Escherichia coli (K12)	Bacteria	β-CT	304	P0A9Q5	NCBI
Arabidopsis thaliana	Brassicaceae	β-CT	488	P56765	NCBI
Auxenochlorella protothecoides	Chlorophyta	β-CT	388	AEU08409	NCBI
Bryopsis hypnoides	Chlorophyta	β-CT	353	ACX33787	NCBI
Chara vulgaris	Chlorophyta	β-CT	293	ABA61965	NCBI
Chlorococcum humicola	Chlorophyta	β-CT	337	ADZ15145.1	NCBI
Chlamydomonas reinhardtii	Chlorophyta	β-CT	369	133238	JGI
Chlorella variabilis	Chlorophyta	β-CT	388	ADZ04992	NCBI
Chlorella vulgaris	Chlorophyta	β-CT	411	BAA57908	NCBI
Coccomyxa subellipsoidea (C-169)	Chlorophyta	β-CT	628	ADV29875	NCBI
Dunaliella salina	Chlorophyta	β-CT	349	ABO33321	NCBI
Helicosporidium sp.	Chlorophyta	β-CT	471	ABD33968	NCBI
Leptosira terrestris	Chlorophyta	β-CT	599	ABO69279	NCBI
Nephroselmis olivacea	Chlorophyta	β-CT	627	AF137379_28	NCBI
Oltmannsiellopsis viridis	Chlorophyta	β-CT	342	ABB81983	NCBI
Oocystis solitaria	Chlorophyta	β-CT	411	ACQ90742	NCBI
Parachlorella kessleri	Chlorophyta	β-CT	441	ACQ90907	NCBI
Pedinomonas minor	Chlorophyta	β-CT	381	ACQ90825	NCBI
Pseudendoclonium akinetum	Chlorophyta	β-CT	312	AAV80676	NCBI
Trebouxia aggregata	Chlorophyta	β-CT	515	ABX82657	NCBI
Trebouxiophyceae sp.	Chlorophyta	β-CT	622	AFQ93852	NCBI
Volvox carteri f. *nagariensis*	Chlorophyta	β-CT	478	82311	JGI
Anabaena variabilis (ATCC 29413)	Cyanobacteria	β-CT	316	YP_320706	NCBI
Microcystis aeruginosa NIES-843	Cyanobacteria	β-CT	316	BAG05575	NCBI
Nostoc azollae (0708)	Cyanobacteria	β-CT	317	ZP_03766652	NCBI
Huperzia lucidula	Lycopodiophyta	β-CT	316	AAT80716	NCBI
Paulinella chromatophora	Rhizaria	β-CT	288	ACB42560	NCBI
Cyanidium caldarium	Rhodophyta	β-CT	267	Q9TLW3	NCBI
Cyanidioschyzon merolae	Rhodophyta	β-CT	273	CMV207C	CMGP
Galdieria sulphuraria	Rhodophyta	β-CT	549	EME28527	NCBI
Gracilaria tenuistipitata var. liui	Rhodophyta	β-CT	293	AAT79592	NCBI
Porphyra purpurea	Rhodophyta	β-CT	288	P51198	NCBI
Porphyra umbilicalis	Rhodophyta	β-CT	288	AFC39868	NCBI
Porphyra yezoensis	Rhodophyta	β-CT	288	Q1XDT9	NCBI
Chaetosphaeridium globosum	Streptophyta	β-CT	297	AAM96498	NCBI
Chlorokybus atmophyticus	Streptophyta	β-CT	296	ABM87949	NCBI
Staurastrum punctulatum	Streptophyta	β-CT	307	AAX45681	NCBI
Zygnema circumcarinatum	Streptophyta	β-CT	297	AAX45794	NCBI