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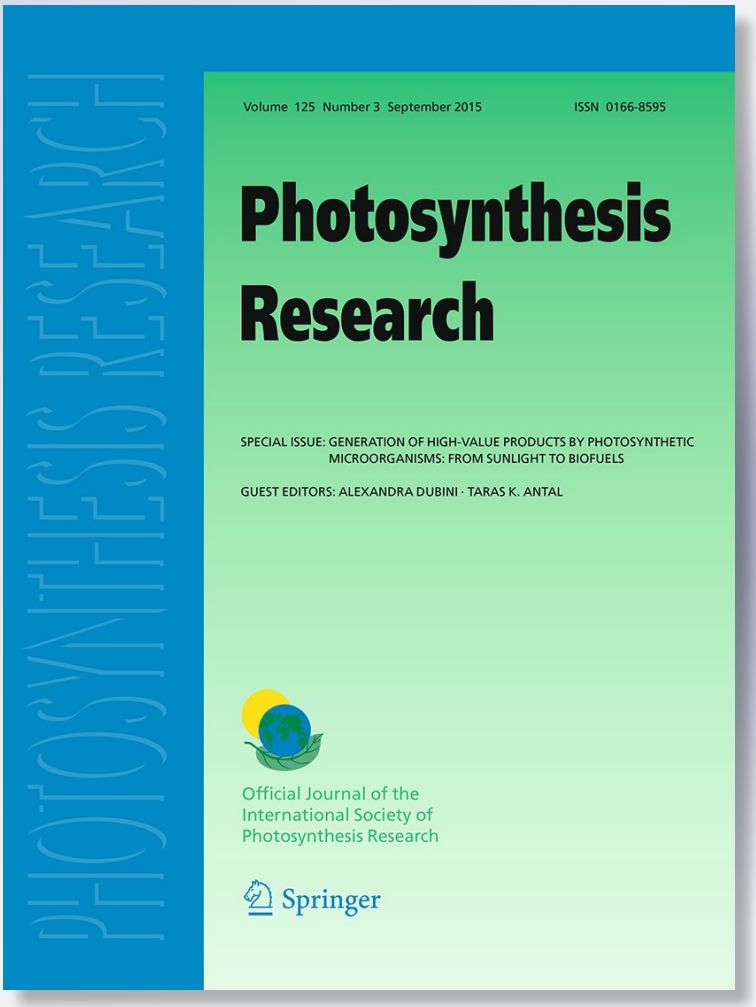
Photosynthesis Research
Official Journal of the International Society of Photosynthesis Research

ISSN 0166-8595
Volume 125
Number 3

Photosynth Res (2015) 125:437-449
DOI 10.1007/s11120-015-0156-3



Springer

Photosynth Res (2015) 125:437–449
DOI 10.1007/s11120-015-0156-3

REVIEW

Recent breakthroughs in the biology of astaxanthin accumulation by microalgal cell

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Received: 30 January 2015 / Accepted: 6 May 2015 / Published online: 15 May 2015

Abstract Massive accumulation of the secondary ketokarotenoid astaxanthin is a characteristic stress response of certain microalgal species with *Haematococcus pluvialis* as an illustrious example. The carotenogenic response confers these organisms a remarkable ability to survive in extremely unfavorable environments and makes them the richest source of natural astaxanthin. Exerting a plethora of beneficial effects on human and animal health, astaxanthin is among the most important bioproducts from microalgae. Though our understanding of astaxanthin biosynthesis, induction, and regulation is far from complete, this gap is filling rapidly with new knowledge generated predominantly by application of advanced "omics" approaches. This review focuses on the most recent progress in the biology of astaxanthin accumulation in microalgae including the genomic, proteomic, and metabolomics insights into the induction and regulation of secondary carotenogenesis and its role in stress tolerance of the photosynthetic microorganisms. Special attention is paid to the coupling of the carotenoid and lipid biosynthesis as well as deposition of astaxanthin in the algal cell. The place of the carotenogenic response among the stress tolerance mechanisms is revisited, and possible implications of the new findings for biotechnological production of astaxanthin from microalgae are considered. The potential use of the carotenogenic microalgae as a source not only of value-added carotenoids, but also of biofuel precursors is discussed.

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Keywords Carotenogenesis · Expression · Ketocarotenoids · Photooxidative stress · Systems stress response

Introduction

Massive accumulation of the secondary ketokarotenoid astaxanthin (Ast) is a characteristic stress response of certain microalgal species (Takaichi 2011) with *Haematococcus pluvialis* (Chlorophyceae) (Sussela and Toppo 2006) as the most illustrious example though this pigment also synthesized by plants, fungi, and bacteria (Goodwin 1961; Johnson and Schroeder 1995). The microalgae capable of massive (upto 4–5 % of cell dry weight, DW) accumulation of carotenoids (Car), including Ast, under stressful conditions are termed as carotenogenic microalgae. In particular, *H. pluvialis* accumulates upto 3–5 % DW Ast (for a comprehensive account of the biology of *H. pluvialis*, reader is referred to the recent reviews by Han (Han et al. 2013a, b) and to the seminal works referenced therein). Astaxanthin is a secondary Car meaning that it is not functionally or structurally coupled to the photosynthetic apparatus (Lemoine and Schoefs 2010; Solovchenko 2013). Accordingly, it does not participate in light harvesting and is exclusively involved in the protection of the algal cells under stressful conditions. The carotenogenic response confers these organisms a remarkable ability to withstand extremely unfavorable environmental conditions and makes them the richest source of natural Ast (Lorenz and Cysewski 2000; Sussela and Toppo 2006; Varshney et al. 2014).

Molecule of Ast features an extensive system of 13 conjugated double bonds making it the most powerful natural antioxidant (Guerin et al. 2003), though it is not yet

clear to which extent the antioxidative effects of Ast are exerted in vivo in the microalgal cell. At the same time, Ast does not exert a prooxidant effect typical of other carotenoids (Otani 2013). Furthermore, Ast is not a vitamin precursor hence its overdose does not pose the threat of hypervitaminosis. Exerting a plethora of beneficial effects on human and animal health (for a recent review, see Yuan et al. 2011), Ast is widely applied as a nutraceutical, pharmaceutical, safe colorant, and aquaculture feed additive (Lorenz and Cysewski 2000; Sussela and Toppo 2006). Reportedly, Ast is the most important Car biotechnologically produced; a number of excellent reviews were dedicated to the commercial aspects of Ast production from microalgae (see e.g., Han et al. 2013b; Leu and Boussiba 2014; Lorenz and Cysewski 2000 and references therein).

Molecule of Ast has two asymmetric carbon atoms at the positions 3 and 3′ of the ionone rings on either end of the molecule. Depending on the hydroxyl groups attached to these carbon atoms, the three possible enantiomers of Ast are designated *R*,*R* (both OH groups are above the plane of the molecule), *S*,*S* (both of the OH groups are below the plane of the molecule), and *R*,*S* (meso-form). The Ast in *H. pluvialis* has optically pure (3S,3′S)-chirality (Renstrom and Liaaen-Jensen 1981), whereas synthetic Ast is a mixture (Su et al. 2014; Wang et al. 2014) of all three isomers containing only 25 % of the biologically active isomers (Yuan and Chen 1998). Nevertheless, over 95 % of the feed market consumes synthetic Ast, which is much more affordable (Johnson and Schroeder 1995; Lorenz and Cysewski 2000; Minyuk et al. 2008). On the other hand, the use of the synthetic pigments, especially in food additives and pharmaceuticals, is less desirable. Therefore, natural Ast remains among the key bioproducts from microalgae despite its high price (generally, ca. 3–4 times higher in comparison with the synthetic Ast) (Guerin et al. 2003; Han et al. 2013a; Lorenz and Cysewski 2000).

Characteristic responses of individual biosynthetic pathways (e.g., those for Car or fatty acids, FA) to the stresses promoting the carotenogenesis are relatively well studied (for recent reviews, see Boussiba 2000; Wang et al. 2014). Still, our understanding of the complex role of the carotenogenic response in stress tolerance of microalgae is incomplete. This is particularly true for early events preceding the massive accumulation of Ast and other manifestations of the acclimation of the carotenogenic microalgae to the stresses. These events involve the sensing of the stress imposed by and the protection of the cell from the buildup of harmful reactive oxygen species (ROS), transient changes in respiration, photosynthetic fixation, and partitioning of carbon between different metabolite pools and cell compartments.

The last several years were marked by explosive growth of the interest to the systems biology of Ast accumulation, orchestration of the pigment biosynthesis by, and its integration into the cell metabolic network under the stress. A number of detailed reports became available dissecting the metabolic changes accompanying massive accumulation of Ast under high light and nitrogen starvation (Chen et al. 2015; Gwak et al. 2014; Recht et al. 2014). As a result, the gaps in our understanding are filling rapidly with new knowledge generated predominantly by application of the advanced "omics" approaches. This review focuses on the most recent progress in the physiology and systems biology of the Ast accumulation in the carotenogenic microalgae with primary emphasis on the most biotechnologically important species, *H. pluvialis* (thought other promising microalgae such as *Chlorella* (*Chromochloris*) *zofingiensis* are also briefly considered). The role of Ast accumulation in stress tolerance of the photosynthetic microorganisms is revisited with the novel genomic, metabolomics, lipidomic, and proteomic insights in mind. We also consider possible implications of the new findings for biotechnological production of Ast from microalgae and for exploiting the enhanced lipid accumulation tightly coupled with the carotenogenic response as a potential source of biofuel precursors.

Astaxanthin accumulation serves multi-level stress protection

Evidently, acclimation and protection against different stresses are served in the "green" (essentially Ast-free motile vegetative cells (Chekanov and Solovchenko 2015)), "brown," and "red" cells (immotile Ast-rich cells, the difference between the latter two will be elaborated on below) of the carotenogenic microalgae by significantly different sets of mechanisms. Thus, the acclimation of photosynthesis in the "green" cells of *H. pluvialis* is accomplished, like in most chlorophyte or higher plant cells, with participation of light harvesting regulation, non-photochemical quenching, and enzymatic ROS elimination systems (Demmig-Adams et al. 2012; Foyer and Shigeoka 2011; Horton 2014). However, these systems are able to cope only with a stress of a limited intensity, hence the "green" cells are more susceptible to the photooxidative damage (Han et al. 2012; Solovchenko 2011).

The transformation of the "green" cells to non-motile palmelloid cells featuring a sizeable amount of Ast on the background of still significant Chl content (so called "brown" cells) and then to the "red" cells is accompanied by a considerable remodeling of the cell protection from (photo)oxidative stress. Consequently, the "brown" cells became generally more tolerant to high light and its

combinations with other stresses in comparison with the "green" cells. As discussed by Wang et al. (2014), the higher stress tolerance of the "brown" cells is determined by different factors (see Fig. 1). First, the "brown" cells are more efficient at avoiding the photosynthetic electron transport chain over-reduction by channeling the excessive photosynthates to the biosynthesis of storage compounds [carbohydrates and eventually neutral lipids (Recht et al. 2012)] and Ast. Second, the linear electron transport in these cells is down-regulated, e.g., by decreasing the level of cytochrome b_6f which may become very low in the "red cells." Third, the excess electron flow generated by PSII can be diverted to a significantly enhanced plastid terminal oxidase (PTOX) or chlororespiration pathway. Accordingly, the important components of the thylakoid membrane including polar lipids and the PSII proteins (particularly, D1 and PsbO) remained relatively stable in the "brown" cells subjected to high light (Han et al. 2012; Wang et al. 2014).

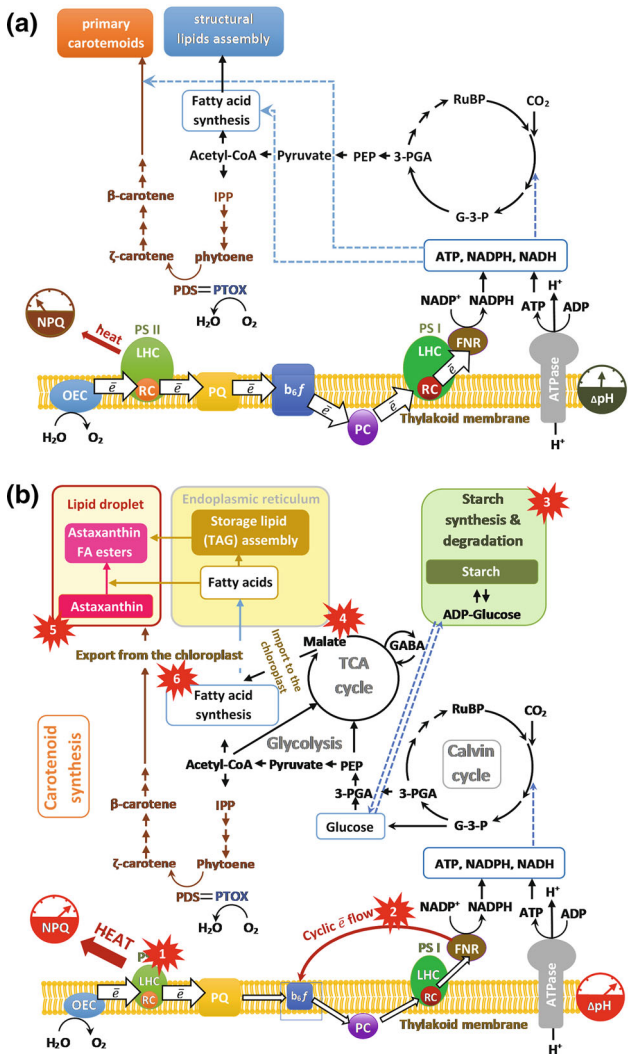

Fig. 1 The schematic view of major changes in photosynthetic electron flow and carbon partitioning patterns of **a** the *green* cells of *H. pluvialis* in the course of the stress-induced accumulation of astaxanthin **b** with an emphasis on lipid and carotenoid synthesis. In the *green* cells, linear electron flow in the electron transport chain of chloroplast dominates, and the fixed carbon mainly partitioned between structural components of the cell and photosynthetic pigments. The onset of carotenogenesis is accompanied by (1) reduction of photosynthetic apparatus and (2) increase of the cyclic electron flow contribution. The photosynthetically fixed carbon as well as metabolites re-partitioned from other metabolic pathways [such as (3) starch turnover and (4) tricarbonic acid cycle] is converted mainly to astaxanthin (5) and (6) fatty acids consumed for the assembly of neutral lipids and esterification of the astaxanthin molecules. Compiled from (Gu et al. 2014; Gwak et al. 2014; Recht et al. 2012; Su et al. 2014)

Still, the cells which did not manage to accumulate sufficient amounts of Ast and retained a sizeable pool of chlorophyll and primary Car may suffer from abrupt and/or prolonged exposition to a stress (e.g., high light, N or P starvation); this is not the case in the "red" cells possessing high Ast content (Gu et al. 2013; Solovchenko 2011). This circumstance has important consequences for biotechnological production of Ast from microalgae. A low stress tolerance of the "green" cells might decline severely the Ast productivity of *H. pluvialis* cultures at the "red" stage due to a high cell mortality (Wang et al. 2014). Accordingly, Wang et al. (2014) suggested that exposing the palmella cells instead of "green" cells to the stress conditions may considerably increase Ast and lipid production in *H. pluvialis* cultures.

Evidently, the transition from the model of photoprotection characteristic of the "green" cells and attaining a high level of stress tolerance is complete only after the formation of Ast-rich "red" cells also called haematocysts. The effects of Ast on the microalgal cell tolerance to different environmental stresses have been studied for more than 20 years so there is a large body of experimental evidence accumulated in the literature, mostly for *H. pluvialis* (see e.g., Hagen et al. 1993b, 1994; Lemoine and Schoefs 2010; Li et al. 2008; Wang et al. 2003 and references therein). It is generally accepted now that Ast accumulation in the course of the carotenogenic response vastly increases cell tolerance to adverse environmental conditions (Boussiba 2000; Lemoine and Schoefs 2010; Wang et al. 2003), but the exact role of Ast in this process is vigorously debated until now. Taken together, the currently available evidence allows to distinguish at least four mechanisms of cell protection by Ast accumulation and metabolic rearrangements (Fig. 1) accompanying this process.

Firstly, Ast is believed to act as an internal sunscreen absorbing excessive light and shielding the chloroplast and other vulnerable cell structures from photoxidative damage (Hagen et al. 1994). Indeed, Ast was shown to be able to intercept in vivo a considerable part of light otherwise

reaching chlorophyll, and the degree of its protection is tightly related with the ratio of the screen (Ast) and the photosensitizer (chlorophyll) in the cell (Hagen et al. 1994; Han et al. 2012; Solovchenko 2011; Solovchenko et al. 2013). Furthermore, the significance of light screening by the lipid droplets (LDs)-containing Ast was further supported by the phenomenon of quick migration of the LD to the periphery of cell in the *H. pluvialis* cells exposed to high light (Peled et al. 2012).

Secondly, the Ast containing LD is suggested to from an antioxidant barrier surrounding the nucleus and the chloroplast and protecting these structures from ROS attacks (Boussiba 2000; Hagen et al. 1993b). Possible antioxidative effect of Ast in vivo has been discussed (Kobayashi 2000; Kobayashi et al. 1997a), but it is unlikely that Ast exerts direct antioxidative effect by elimination of the ROS in the thylakoid membranes because this pigment is accumulated in cytoplasmic LDs, which are distant from the sites of ROS generation in the chloroplast. On the other hand, Ast was found to be capable of binding to the photosynthetic pigment–protein complexes and to accumulate within the thylakoid membranes in transgenic plants (Röding et al. 2015). Currently, it seems more likely that the protection of the cell at the initial phase of the stress-inducing Ast accumulation is performed by the "classic" antioxidative enzymes superoxide dismutase, catalase, and peroxidase (Wang et al. 2004, see also Fig. 2). These enzymes generally undergo transient up-regulation before the onset of the gross accumulation of Ast, usually within the first 2 days of the stress exposure. There are also some clues from the recent metabolomic studies (Su et al. 2014) pointing to the possible involvement of the molecules with antioxidative function such as thioredoxin(s) and glutathione in the stress response of *H. pluvialis*. The putative antioxidative role of Ast is expected to be significant in the LD where Ast can protect the lipids containing unsaturated FA prone to peroxidation.

Thirdly, biosynthesis of Ast consumes, as the substrate of β-carotene hydroxylase and ketolase (see below), O_2 potentially dangerous for the cell under stress. The relative decline in steady-state O_2 concentration in the cell during vigorous Ast accumulation is estimated to be above 10 % of its stationary concentration which may be significant under adverse conditions (Li et al. 2008). Molecular oxygen is also reduced to H_2O in the course of carotenoid biosynthesis by the enzyme plastid terminal oxidase (PTOX), the co-factor of phytoene, and ζ-carotene desaturases (Bennoun 2001; Li et al. 2010) (Fig. 1); for a comprehensive account of PTOX roles in phototrophic cell, see the recent review by Nawrocki et al. (2015). It was also shown that the induction of Ast biosynthesis is coordinated with the up-regulation of the expression of the genes of antioxidative defense (Gwak et al. 2014; Kim et al. 2011).

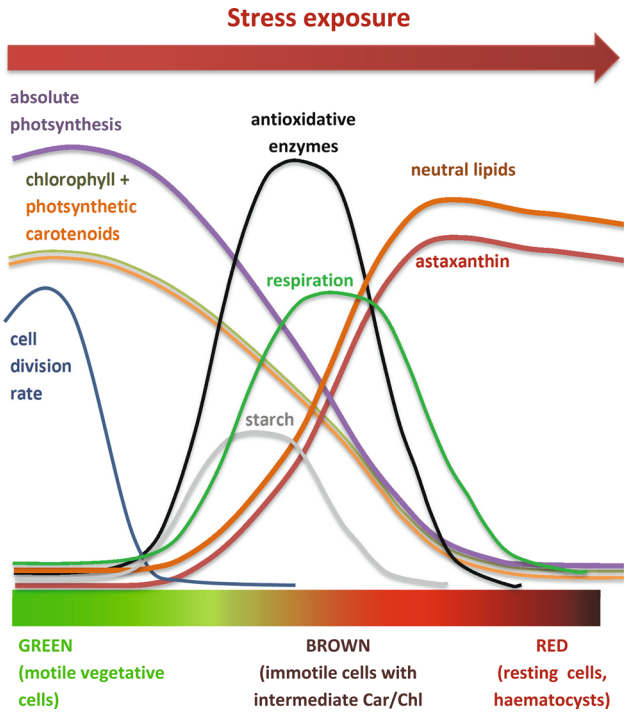

Fig. 2 A hypothetic scenario of the physiological changes accompanying the induction of astaxanthin accumulation in the stressed cells of *H. pluvialis*. At the initial stages of the astaxanthin accumulation, the cell retains a significant amount of photosynthetic pigments and photosynthetic activity driving accumulation of starch. At this stage, the up-regulated antioxidative enzymes protect the cell. Later, the starch synthesis is followed by its degradation, a rise of respiration rate and induction of fatty acid and astaxanthin biosynthesis resulting in the appearance of red lipid droplets in the cell. Decline in photosynthetic pigments proceeds (although the specific photosynthesis rate might be preserved), the antioxidative enzyme activity reverts to the basal level

Finally, the biosyntheses of Ast and FA (see the section "Coupling of astaxanthin and lipid biosyntheses" below) provide a potent sink for the photosynthates that cannot be utilized for cell growth and division under the stress. This sink further mitigates the risk of damage by ROS increasingly accumulated when the electron carriers in the chloroplast electron transport chain are over-reduced (Han et al. 2012).

Massive astaxanthin accumulation and photosynthesis

It is conceivable that biosynthesis of Ast and lipids of LD as well as maintenance of the cell homeostasis under the stressful conditions should generate a considerable demand of energy and building blocks for the carotenoid and lipid biosynthesis. A significant level of metabolic activity during the initial phase of carotenogensis is thought to be

necessary for a successful transformation of the "green" cell to Ast-rich "red" cells (Gwak et al. 2014). On the other hand, previously obtained physiological evidence and the recent analysis of the gene expression pattern showed that the formation of the "red" cells is accompanied by down-regulation of photosynthesis and enzymes responsible for biosynthesis of chlorophyll, LHC (Gu et al. 2014; Gwak et al. 2014), and enzymes of carbon fixation (Kim et al. 2011). This is consistent with the observed reduction of chloroplast, decomposition of the grana and lamellae system (Gu et al. 2013; Peled et al. 2012), probably to reduce the production of ROS in the cell (Wang et al. 2004).

However, recent evidence indicates that a considerable part of photosynthetic activity is retained during the onset and subsequent progress of the carotenogenic response in spite on the profound decomposition of thylakoids. Thus, Gu et al. (2013) argued that even fragmented thylakoid membranes are able to maintain a moderate level of photosynthetic activity in *H. pluvialis* cells. This seems to be possible since the initial phase of Ast accumulation is accompanied only by a transient down-regulation of D1 protein of PSII although without a sizeable decline in the rate of photosynthetic oxygen evolution (Wang et al. 2003), and a certain amount of chlorophyll and accessory pigments is also retained even at the most advanced stages of Ast accumulation. Hagen et al. (1993a) also detected an increase in the transthylakoid proton gradient along with oscillations in non-photochemical fluorescence quenching and attributed this to active CO_2 fixation in the course of "red" cell formation.

Based on the measurements of respiration rates and metabolic activity of the "red" *H. pluvialis* cells, it was suggested that insufficient energy supply by photosynthesis under stressful conditions could be compensated by elevated respiration and glycolysis processes (Hagen et al. 1993b; Recht et al. 2012, 2014). This suggestion is compatible with the transient up-regulation of mitochondrial respiratory proteins after the onset of Ast-inducing stress (Wang et al. 2004). The increased demand in ATP could also be satisfied by increased phosphorylation as a result of up-regulation of cyclic electron transport over PS I and overall increase in the PS I to PS II activity ratio (Hagen et al. 1993a).

Key steps of asthaxanthin biosynthesis

The biochemistry and enzymology of Ast biosynthesis as well as its genetic control are relatively well studied and extensively reviewed elsewhere (Cunningham and Gantt 1998; Han et al. 2013b; Lemoine and Schoefs 2010; Nisar et al. 2015; Takaichi 2011) so the key steps of Ast biosynthesis are just briefly recapitulated here. More attention is paid to the recently discovered metabolic constrains and regulatory events accompanying the induction and progress of Ast accumulation in microalgae (see the next section).

As in higher plants, the precursor of Car in Chlorophyta is isopentenyl pyrophosphate (IPP, C_5) originating from glycerophosphate-pyruvate (non-mevalonate or 1-deoxy-D-xylulose-5-phosphate, DOXP) pathway, whereas in Euglenophyceae, it is formed via mevalonate pathway (Han et al. 2013b; Lichtenthaler 1999; Zhao 2013). Direct evidence on the gene level were recently obtained for the involvement of the DOXP pathway in IPP biosynthesis in *H. pluvialis* (Gwak et al. 2014). The enzyme IPP isomerase (IPI, encoded in *H. pluvialis* by oxidative stress-induced *Ipi1* or translation-regulated *Ipi2* gene (Sun et al. 1998)) converts IPP to dimethylallyl pyrophosphate (DMAPP). Successive attachment of three IPP molecules to a DMAPP molecule by geranylgeranyl pyrophosphate (GGPP, C_{20}) synthase yields the molecule of GGPP. Two GGPP molecules undergo head-to-tail condensation to form phytoene in the reaction catalyzed by phytoene synthase (PSY, encoded by the gene *Psy*) (Cunningham and Gantt 1998).

Phytoene molecule is the precursor of all carotenoids. In four sequential desaturation reactions catalyzed by phytoene desaturase (PDS, *Pds1*) and ζ-carotene desaturase (ZDS, *Zds1*), it is converted sequentially to phytofluene, ζ-carotene, neurosporene, and lycopene (5, 7, 9, and 11 conjugated double bonds, respectively) (Cunningham and Gantt 1998). PTOX is a plastoquinol oxidase serving as a co-factor of the desaturases and the key oxidase in chlororespiration (Nawrocki et al. 2015). Wang et al. (2009) cloned and sequenced two PTOX cDNAs from *H. pluvialis*, designated as *ptox1* and *ptox2* participating in Ast synthesis and playing a critical protective role against stress by reducing O_2 to H_2O (Bennoun 2001; Li et al. 2010). Comparative analysis of the transcriptional expression of *ptox1*, *ptox2,* and *pds* indicated that the up-regulation of PTOX1 but not PTOX2 is correlated with Ast synthesis (Wang et al. 2009). The membrane-bound enzyme lycopene β-cyclase (LCYB, *LcyB*) sequentially converts the linear molecule lycopene to β-carotene possessing two β-ionone rings (Cunningham and Gantt 1998).

All steps mentioned above take place in the chloroplast. It was shown that β-carotene is the Ast precursor which is exported from the cytoplasm (Grünewald and Hagen 2001), and the remaining steps of Ast formation (see the section "Lipid droplets: factories and subcellular depots of astaxanthin") occur in the LD subcompartment. The mechanism(s) of β-carotene transport to the LD remains so far elusive: though the simultaneous presence of β-carotene and Ast in *H. pluvialis* LD was confirmed by Raman microspectrometry (Collins et al. 2011), electron-microscopic examination did not reveal any structures related to the transport of β-carotene (Grünewald and Hagen 2001).

The conversion of β-carotene to Ast requires introduction of two hydroxyl groups (in the positions 3 and 3′) and two keto-groups (in the positions 4 and 4′). The former reaction is catalyzed by 3,3′-hydroxylase CRTR (encoded by *ChyB*), and the latter by 4,4′-ketolase CRTO (*CrtO*) or BKT is represented by several forms, *Bkt1–Bkt3*, in different *H. pluvialis* strains (Grunewald et al. 2001). Under stress, multiple *BKT* genes are up-regulated, and upon reaching a certain threshold level of *BKT* transcripts, *H. pluvialis* begins to massively synthesize Ast (Huang et al. 2006).

A large body of evidence reviewed by Lemoine and Schoefs (2010) suggests that the addition of keto-groups occurs before the hydroxylation and the formation of Ast from β-carotene in *H. pluvialis* proceed most likely (and predominantly) via echinenone (one keto-group), canthaxanthin (two keto-groups), and adonirubin (two keto- and one hydroxygroup). At the same time, this is not necessarily the case in all Ast-accumulating microalgae. Indeed, a BKT from *H. pluvialis* (BKT3) expressed in *E. coli* exhibited the lowest efficiency of the conversion of zeaxanthin to Ast, whereas analogous enzymes from *C. reinhardtii* (CrBKT) and *Chlorella zofingiensis* (CzBKT) were much more efficient (Zhong et al. 2011).

Coupling of lipid and astaxanthin biosyntheses in the course of carotenogenesis: source of value-added carotenoids and biofuel precursors

As was earlier established by Zhekisheva et al. (2002), accumulation of Ast is tightly related with the biosynthesis of FA such as oleic acid associated predominantly with triacylglycerols. This class of neutral lipids is the major constituent of cytoplasmic LD forming the intracellular depot for Ast (see the "Lipid droplets: factories of and subcellular depots for astaxanthin" section below). Fatty acids are also consumed for esterification of polar hydroxyl groups of Ast prior its deposition within the hydrophobic environment of the LD. Accordingly, inhibition of the lipid biosynthesis abolished the accumulation of Ast, whereas blocking Ast biosynthesis did not prevent the accumulation of neutral lipids and formation of LD (Zhekisheva et al. 2005). The results obtained by Chen et al. (2015) disproved possible coordination of lipid and Ast biosynthesis at the transcriptional level and confirmed that this interaction was feedback related at the metabolite level. The in vivo and in vitro experiments of these authors indicated that Ast esterification by a specific diacylglycerol acyltransferase is the process driving the formation and accumulation of Ast.

The bulk of Ast in *H. pluvialis* is in the form of mono- and diesters of palmitic (16:0), oleic (18:1), or linoleic (18:2) FA. The composition of Ast esters in *H. pluvialis* red cysts comprises ca. 70 % monoesters, 25 % diesters, and 5 % of the free ketocarotenoid (Johnson and Schroeder 1995; Zhekisheva et al. 2002). Fatty acid content in the "red" cell lipids of *H. pluvialis* can be as high as 30–60 % of DW with >80 % unsaturated FA (Goncalves et al. 2013). As argued by Damiani et al. (2010), the capacity of *H. pluvialis* to accumulate high (upto 32.99 % of the cell dry weight) amounts of the neutral lipids incorporating predominantly moderately unsaturated FA from the families C16 and C18 in response to the stresses makes it a potential oil-enriched feedstock for biodiesel production. Indeed, there were attempts to estimate the suitability of *H. pluvialis* biomass for the conversion to biodiesel (Damiani et al. 2010). However, nowadays, the use of *H. pluvialis* biomass for the extraction of Ast is much more economically viable than the production of biodiesel. Still, the bulk lipid and biomass waste remaining after extraction of Ast can be valorized via conversion to biodiesel and/or other kinds of biofuels. In this case, the Ast remaining in the lipid extract or biomass could serve as a natural antioxidant protecting the biofuel against oxidative degradation making it more suitable for long-term storage.

A model was recently developed integrating the stress responses of Ast biosynthesis, carbohydrate, and lipid metabolism in *H. pluvialis* (Recht et al. 2012, 2014). The up-regulation of the Ast biosynthesis takes place on the background of the transient induction of carbohydrate accumulation (Recht et al. 2014). Metabolic profiling showed that as long as Chl content is relatively high (>12 mg L^{-1} which is the case in the "brown" cells), the cell invests its carbon and energy to the pool of free sugars and starch. Further exposure to stress causes a transition to degradation of previously accumulated carbohydrates (Recht et al. 2012) and synthesis of fatty acids (Recht et al. 2014), as in different chlorophyte species (Goncalves et al. 2013; Gorelova et al. 2014).

The advanced stages of stress-induced carotenogenesis when the rate of Ast biosynthesis slows down (Recht et al. 2014) might be accompanied by a shutdown of central metabolism (judging from ATP and other NTPs as well as ribulose bisphosphate levels) except the pathways responsible for FA biosynthesis de novo (as reflected by acetyl-CoA, dihydroxyacetone phosphate, and 3-phosphoglycerate levels; see also Fig. 1). The conspicuous metabolic changes during the induction Ast accumulation also involve the increase in the pool of acetyl-CoA, a key precursor of FA synthesis, and the stearic and palmitic acids (Su et al. 2014) which are the major FA esterifying Ast (Zhekisheva et al. 2002). The up-regulation of other genes related with FA synthesis such as alkane 1-monooxygenase, alcohol dehydrogenase, and triacylglycerol lipase took place under stress conditions including both nutrient starvation and high irradiance (Kim et al. 2011).

The recent findings by Recht et al. (2014) are also indicative of the relationship between the carotenogenesis and the increased activity of the tricarboxylic acid (TCA) cycle. It was suggested that TCA cycle plays a key role in FA biosynthesis under stressful conditions conductive for the carotenogenesis furnishing excess malate, which could support FA biosynthesis after import to the chloroplast (Fig. 1b). This is compatible with the increase in respiration-related transcripts (i.e., glycolysis, TCA cycle, electron transport, phosphorylation) during Ast accumulation in *Haematococcus* (Kim et al. 2011). Using inhibitor analysis, Recht et al. (2014) confirmed the involvement of malic enzyme in the induction of fatty acid biosynthesis accompanying the carotenogenic stress response and proposed the existence of a significant flux of malate to the chloroplast with its subsequent involvement in de novo FA biosynthesis. In contrast to *Chlamydomonas reinhardtii* (Shtaida et al. 2014), it is unlikely that in the stressed *H. pluvialis,* a high amount of carbon flows from pyruvate to Acetyl-CoA, and continuous carbon fixation (see the "Massive astaxanthin accumulation and photosynthesis" section) suggests that pyruvate flows into other pathways, e.g., the non-mevalonate pathway for the biosynthesis of Car.

Lipid droplets: factories of and subcellular depots for astaxanthin

It is conceivable that secondary Car including Ast cannot accumulate in appreciable amounts within the thylakoid membranes (which composition is under strict genetic control) or, in the free form, within the hydrophilic environment of the cytoplasm. Indeed, the presence of Ast in the chloroplast membranes of the transgenic tobacco leads to destabilization of its lipid phase and PS II supercomplexes (Röding et al. 2015). The massive accumulation of Ast under stress turns feasible in the presence of dedicated subcellular structures—cytoplasmic LD forming a potent sink for Ast, the end product of the corresponding pathway, avoiding the feedback inhibition of the Car biosynthesis. It was conclusively demonstrated that formation of the LD subcompartment in the cell is among key factors driving (Zhekisheva et al. 2002) and limiting (Zhekisheva et al. 2005) accumulation of Ast in the cells of carotenogenic microalgae (Lemoine and Schoefs 2010). This is one of the reasons making massive accumulation of secondary Car more feasible from the standpoint of biotechnology in comparison with overproduction of primary Car (e.g., lutein and fucoxanthin). The latter is more problematic since it will require modification of gene expression or enzyme activity, most likely combined with the creation of storage structures outside of the photosystems (Mulders et al. 2014b).

The exact mechanism of LD formation is so far not known though it is commonly accepted that the globules are formed from vesicles budding from the endoplasmic reticulum (Guo et al. 2009; Murphy 2001b). This mechanism is more similar to that common for a different cell types including bacterial, higher plant, and animal cells, whereas the plastidial lipid globules of *Dunaliella salina* accumulating β-carotene are more closely related to stigma-like structures (Davidi et al. 2014).

The bulk (>90 % of total FA content) of the inner LD matrix is formed by neutral lipids, mainly triacylglycerols (TAG), polar phospholipids (PC), sulfolipids (SQDG), and glycolipids (mainly MGDG) as well as betain lipids (DGTS) constituting monolayer LD membrane and specialized amphifilic proteins (Murphy 2001a; Peled et al. 2011). The major FA in the lipids of LD are palmitic (16:0), oleic (18:1), and linoleic (18:2) acids (Peled et al. 2011; Zhekisheva et al. 2002).

The major LD-forming protein in *H. pluvialis* (*Haematococcus* oil globule protein, HOGP) was 275-amino acid long 33 kDa protein partially homologous to *Ch. reinhardtii* oil globule protein. This protein was hardly detectable in vegetative cells but increased more than 100-fold within 12 h of nitrogen deprivation and/or high light stress exposure (Peled et al. 2011). The major LD-associated proteins of green microalgae are thought to be encoded by a novel gene family specific to Chlorophyta. The globule-associated protein biosynthesis might be regulated at the translational or post-translational level to sustain the biogenesis and enormous accumulation of the LD (Gwak et al. 2014).

As noted above, relatively polar Ast molecules are esterified by fatty acids before deposition in the LD. As reported by Chen et al. (2015), Ast esterification occurs in the endoplasmic reticulum. Thus, in *H. pluvialis* and Ast-accumulating representatives of the genus *Chlorella* during the final steps of cyst formation, more than 95 % of Ast is converted to fatty acid esters (Sussela and Toppo 2006). There are controversial reports on the Car composition of LD in *H. pluvialis*: biochemical methods reveal only Ast in the LD (Peled et al. 2011), whereas Raman microspectrometry is evident of the simultaneous presence of *β*-carotene and Ast in *H. pluvialis* LD (Collins et al. 2011). As shown by nonlinear optical microscopy imaging, the Ast accumulated within the LD of *H. pluvialis* is characterized by highly ordered isotropic packaging; this is in contrast to the highly anisotropic organization of Ast in synthetic aggregates (Tokarz et al. 2014). This might be an additional indication of the involvement of the enzymes associated with the LD membranes in the biosynthesis of the Ast molecules.

Recent studies emphasized another important role of LD as a structure where the final steps of Ast biosynthesis take

place. Therefore, it is possible to think that blocking the stress-induced biosynthesis of lipids and LD formation abolishes the accumulation of Ast not only due to the lack of the sink for Ast but also due to disturbance of the oxygenation of β-carotene as well. This is not typical for either higher plants or microalgae: thus, the β-carotene-containing globules of *Dunaliella salina* are situated in the chloroplast and do not participate in the carotenoid biosynthesis (Davidi et al. 2014, 2015). Interestingly, the literature available at the time of this writing lacked reports on the detectable accumulation of Ast precursors in the LD compartment.

The evidence of participation of the LD in Ast biosynthesis stems from inhibitory and immunocytochemical analyses showing that in *H. pluvialis*, CRTO is present not only in chloroplasts but also in the LD (Grunewald et al. 2001). As also noted by Grünewald et al. (2001), CRTO molecules are observed not only in the LD periphery but also inside these structures, which is in agreement with hydrophobic properties of this enzyme. In fact, CRTO is active in the hydrophobic surrounding, such as TAG matrix of the LD. It is likely that in such medium, CRTO is protected from the protease attack, which may explain continued enzyme activation on the background of a decrease in its mRNA content. It is worth mentioning that, in spite of CRTO presence inside LD, its molecules are active only on the OS surface. Apparently, this is explained by the requirement of co-factors coming from other compartments, such as the endoplasmatic reticulum and Golgi apparatus, and this suggestion is supported by co-localization of OS and these structures (Grunewald et al. 2001).

Induction and regulation of massive astaxanthin accumulation

Generally, accumulation of secondary Car including Ast in microalgal cells takes place under adverse conditions slowing down the cell division and photosynthesis, e.g., under excessive irradiance, nutrient deficiency, extreme temperatures, salinity, and their combinations (Boussiba 2000; Solovchenko 2013). Different stresses inducing accumulation of Ast affect the expression of approximately the same subset of the genes controlling Car biosynthesis. Thus, the high light and salinity stresses simultaneously up-regulate the transcription of many genes starting from the formation of IPP and including β-carotene hydroxylase (see the section "Key steps of astaxanthin biosynthesis") (Gao et al. 2014; Kim et al. 2011; Mulders et al. 2014b). The level of *CrtR-b* transcripts and Ast accumulation was linearly related to the *H. pluvialis* wild type and the Ast-hyper-accumulating mutant (MT 2877), suggesting a transcriptional control of *CrtR-b* over Ast biosyn-thesis (Li et al. 2008, 2010). On the other hand, the amount of BKT protein increases in parallel with accumulation of the corresponding *bkt* transcript only during the early steps of Ast synthesis, whereas a further enzyme accumulation is not accompanied by a proportional increase in the mRNA amount. This may indicate also the involvement of not only transcriptional, but also (post)-translational mechanisms in the regulation of this enzyme activity (Grunewald et al. 2000).

Li et al. (2010) demonstrated that most of the Car synthesis genes are up-regulated at the transcriptional level in response to high light, excess ferrous sulfate, and excess sodium acetate. Interestingly, the combination stress (e.g., high light + salt stress + iron stress) induced lower initial levels of the gene transcripts in comparison with the individual stresses which, after a certain lag, exceeded the levels of the individual stresses (Li et al. 2010). It is important to note that carotenogenic response serves the long-term protection and survival of the cell under extended exposure to the stress, whereas the 'classical' enzymatic defense systems mainly serve as short-time defense mechanisms in *H. pluvialis* (Wang et al. 2004, see also Fig. 2). This might also explain a delayed expression of the carotenogenesis genes with greater expression levels occurred in the cul-tures treated with the multiple stressors (Li et al. 2010).

The authors hypothesized that *H. pluvialis*, like other microalgae, possess both 'generic' (independent on the nature of the stressor) and stressor-specific defense mechanisms. In this case, the carotenogenic response is largely induced by high light, whereas the other defense reactions may be triggered by salinity or excess iron ion. Should this be the case, the combination stress would activate, in a coordinated manner, different protection mechanisms with different efficiency of the induction of carotenogenesis. On the other hand, there are reports suggesting that most of the stresses are essentially interchangeable regarding the induction of Ast accumulation (Kobayashi et al. 1993, 1997b).

The stresses are thought to be sensed by the photosynthetic cells via a number of putative sensors such as plastoquinone pool reduction state, membrane fluidity, stationary concentration of ROS and/or some metabolic 'hub' enzymes (Huner et al. 2012). Thus, a common effect of the environmental stresses is the increase of stationary concentration of ROS in the cell (Foyer and Shigeoka 2011; Sirikhachornkit and Niyogi 2010). On the other hand, there is ample evidence suggesting the involvement of ROS, probably as secondary messengers, in the induction of Ast accumulation (Yong and Lee 1991). The role of ROS as the messengers responsible for the crosstalk between different stimuli is evidenced by treatment of the microalgal cells with ROS generators, e.g., H_2O_2 which induces accumulation of Ast even in the absence of the

other stimuli mimicking the action of environmental stressors; on the contrary, ROS scavengers suppress massive accumulation of Ast (Kobayashi et al. 1997a).

Supplementation of the microalgal culture with organic carbon sources such as acetate or sugars causing feedback inhibition of photosynthetic carbon fixation (but not for primary photochemistry) is also conductive for Ast accumulation (Kobayashi 2000; Kobayashi et al. 1993). These findings are compatible with suggestion that biosynthesis of Ast is regulated by plastoquinone pool as a redox sensor (Steinbrenner and Linden 2003) which is mostly reduced under the stressful conditions when the light absorption exceeds the energy utilization capacity of dark reactions of photosynthesis (Lemoine and Schoefs 2010). In addition, the over-reduced electron carries in the chloroplast electron transport chain might further augment the ROS pool in the stressed algal cells.

An interesting research avenue is represented by studies of phytohormone stimulation of Ast accumulation by microalgal cells (Gao et al. 2013a, b). In the *H. pluvialis* cells, gibberllin A3 (GA3) treatment differentially up-regulated the genes *Ipi-1*, *Ipi-2*, *Psy*, *Pds*, and *Bkt2* in a concentration-dependent manner. Thus, GA3 in concentration 20 mg L^{-1} had a greater effect on the expression of *Bkt2,* whereas 40 mg L^{-1} of GA3 leads to a stronger up-regulation of *Ipi-2*, *Psy*, and *Bkt2*. The expression of *Lyc*, *CrtR-B,* and *CrtO* was not affected by GA3 (Gao et al. 2013a). Another plant hormone, epibrassinolide (EBR) increased, in the concentration range 25–50 mg L^{-1}, Ast productivity via up-regulation of the eight genes of the carotenoid biosynthesis pathway at the transcriptional (*Pds*, *Lyc*, *CrtR-B*, *Bkt*, and *CrtO*), post-transcriptional (*Ipi-1* and *Psy*) or both levels (*Ipi-2*) (Gao et al. 2013b). The comparative studies of the phytohormone effects in the microalgae and higher plants are expected to reveal new information about the regulation of the carotenogenesis. Exogenous application of GA3 and EBR is also considered as a promising way of enhancement of Ast productivity of *H. pluvialis* mass cultures.

Is there a way for increasing microalgal astaxanthin productivity?

As reviewed by Mulders et al. (2014b), microalgae have a number of advantages as a source of natural compounds, including Ast, since these organisms (i) selectively accumulate the pigment of interest (up to ca. 95 % of the total carotenoids) in the amounts by far exceeding those of higher plants; (ii) grow rapidly; (iii) do not compete with crops for arable land. Currently, *H. pluvialis* is the most biotechnologically significant microalgal producer of Ast despite of its intrinsic shortcomings such as slow growth rate, low biomass yield, high risk of contamination at the "green" stage, and a high light requirement for the induction of Ast biosynthesis. Another highly promising candidate species is represented by *C. zofingiensis* (Mulders et al. 2014a, 2015). Its advantages include faster growth rate in a phototrophic, heterotrophic, or mixotrophic cultures, also at ultrahigh cell densities (Han et al. 2012; Liu et al. 2014).

However, the cost efficiency of current microalgal biotechnologies for production of Ast from microalgae is severely limited by productivity of known strains (Lorenz and Cysewski 2000). Indeed, only a limited number of microalgal species (apart from *H. pluvialis*), exclusively from Chlorophyta, are capable of synthesizing Ast. A comprehensive list compiled by Han et al. (for more detail, see Han et al. 2013a and references therein) includes *Botryococcus braunii* (0.01 % DW), *Chlamydocapsa spp.* (0.04 % DW), *Chlamydomonas nivalis*, *Chlorococcum* sp. (0.7 % DW), *Chloromonas nivalis* (0.004 % DW), *Eremosphera viridis*, *Neochloris wimmeri* (1.9 % DW), *Protosiphon botryoides* (1.4 % DW), *Scenedesmus* sp. (0.3 % DW), *Scotiellopsis oocystiformis* (1.1 % DW), and *Trachelomonas volvo-cina*. More recent addition to this list comprises *Bracteacoccus minor* (Minyuk et al. 2014) and *Euglena sanguinea* (G. Minyuk, personal communication). Even more limited number of the chlorophyte species is able to overproduce Ast, i.e., accumulate this pigment in biotechnologically significant amounts (4–5 % DW) under the stressful conditions (Liu et al. 2014).

One may expect a certain increase in Ast productivity from optimization of cultivation conditions. Considering the two-stage cultivation approach commonly applied for production of Ast from *H. pluvialis* [for an account of the biotechnology of Car from microalgae, see (Del Campo et al. 2007; Han et al. 2013b; Leu and Boussiba 2014; Lorenz and Cysewski 2000; Solovchenko and Chekanov 2014)], this will presume (i) increasing the biomass productivity at the "green" stage and (ii) further optimization of the cultivation conditions. However, this avenue of increasing algal productivity is highly explored and exploited already (Mulders et al. 2014b) so it is unlikely to produce a revolutionary increase in Ast productivity.

A promising way of achieving Ast accumulation above the 5 % DW threshold [which is a prerequisite for favorable competition of the natural Ast from microalgae with the synthetic pigment (Del Campo et al. 2007)] is offered by microalgal strain engineering. One of the approaches is strain manipulation at the regulatory level aiming at the removal of possible bottlenecks of Ast biosynthesis. Generally, to increase the metabolic flux towards the biosynthesis of the pigment of interest, one needs to over-express the enzyme(s) that exert control over the flux towards the desired pigment (or replace/augment the rate-limiting enzymes by more efficient analogs from different organisms),

without much disturbance to the rest of the metabolic network of the cell (Farré et al. 2014, 2015). However, one should beware of competition between secondary and primary carotenoid biosyntheses which will most likely result in growth inhibition. Possible targets of this approach include the enzymes catalyzing rate-limiting steps in the biosynthesis of Ast, e.g., phytoene desaturation and lycopene cyclization (Lemoine and Schoefs 2010) as well as ketolation of β-carotene (Han et al. 2013a). The strains with increased Ast accumulation capacity are characterized by the early up-regulation of the genes controlling the rate-limiting steps of the carotenoid biosynthesis, e.g., *psy*, *lyc*, *bkt2*, *crtR-B,* and *crtO* (Gao et al. 2014). This finding may provide a hint for selection of the target genes for up-regulation. On the other hand, the increased transcript level of these genes is not correlated to the different Ast accumulation levels so the expression patterns of these genes are likely strain specific (Gao et al. 2014).

Still, metabolic pathways in microalgae are complex, with numerous regulatory inputs and interplay with other pathways so it is difficult to find the best manipulation target(s) and predict the outcome with confidence (Farré et al. 2014, 2015). In the case of Ast, this approach is complicated at least by (i) distribution of the enzymes of Ast biosynthesis between compartments (chloroplast and LD), (ii) a potential bottleneck associated with the export of β-carotene from the chloroplast to the LD which mechanism remains so far elusive. Remarkably, a sufficiently developed transformation method and genetic toolbox were developed recently (Sharon-Gojman et al. 2015). Still, advanced knowledge-based metabolic engineering based on large "omics" dataset mining opens the way for a precise identification of relevant genes and regulatory mechanisms yielding models that predict the most suitable manipulation points. More sophisticated strategies based on fine-tuned transgene expression, thermodynamic, and kinetic models will be then necessary to balance the metabolic fluxes in the entire Ast biosynthesis pathway [for a comprehensive account of the metabolic engineering strategy, see e.g., (Farré et al. 2015)].

Concluding remarks

Recent insights unveiled the amazing picture of the carotenogenic response as a sophisticated, precisely regulated and tightly integrated into the metabolic network mechanism for coping with diverse stresses. As a result, a number of paradigms related with the role of Ast in the stress tolerance of phototrophs were gradually changed. It is now clear that Ast biosynthesis is not mere an isolated branch of secondary carotenoid biosynthesis. Its integration with central metabolism (especially with carbohydrate and fatty acid biosyntheses) is much more complex (Gwak et al. 2014; Recht et al. 2014) than it was suggested earlier. Another striking finding originated from the comparative transcriptomic and lipidomic analysis is that the Ast-rich resting cells of *H. pluvialis* are probably not quiescent at all but instead are more metabolically active than the motile vegetative "green" cells (Gwak et al. 2014). Then, the cytoplasmic lipid droplets (LDs) serving as the depot for Ast synthesized during "green" to "red" cell transition appear to be much more than just a intracellular reservoir for the lipids and carotenoids. On the contrary, LD plays a key role in the biosynthesis of Ast (Grunewald et al. 2001; Lemoine and Schoefs 2010; Peled et al. 2011) and participate in active protection of the microalgal cell (Peled et al. 2012).

Further significant increase in Ast content for currently known native strains by means of cultivation condition adjustment does not seem to be realistic now. At the same time, the natural algal diversity, which is so far underexplored, may well surprise us with yet unknown strains with high carotenogenic capacity. At the same time, the limitations of native organisms such as the limitation imposed by the capacity of the intracellular Ast depots may be overcome, with time, using engineering approaches. In particular, expansion of the LD subcompartment would enhance this sink for Ast eventually improve its accumulation. Probably, this goal can be achieved via engineering for the enhanced lipid production using one of the techniques recently developed for the improvement of biofuel producing strains (Rosenberg et al. 2008; Trentacoste et al. 2013).

It is generally (and rightfully) believed now that the direct use of Ast-rich microalgal biomass for production of biofuel is not economically viable. On the other hand, there are largely unexplored possibilities of turning the waste generated from the processing of Ast-rich biomass and purification of Ast for human consumption to valuable bioproducts including different kinds of biofuels. In particular, the neutral lipids co-accumulated with Ast turned to be highly suitable for conversion to biodiesel (Damiani et al. 2010).

Finally, despite the significant progress outlined above, our understanding of the biology of Ast in the microalgal cell is still far from complete. In particular, much more work is needed to decipher the machinery of the Ast transport between the chloroplast and LD compartments. Another enigmatic process is Ast catabolism presumably taking place in the cells reverting from the "red" to the "green" stage. Nevertheless, should the investigation of the carotenogenesis and the related processes in the cell remain as vigorous as they are now, there would be a good chance to find the missing answers in the nearest future.

Acknowledgments Helpful notes of Mr. Konstantin A. Chekanov are greatly appreciated.

Conflict of interest The author declares that he has no conflict of interest.

Funding This study was funded by Russian Scientific Fund (project# 14-50-00029).

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