Chapter 2
Brassinosteroids in Microalgae: Application for Growth Improvement and Protection Against Abiotic Stresses

Andrzej Bajguz

Abstract Brassinosteroids have been found in a broad spectrum of microalgae, their biological activities correspond to the function in higher plants. Studies on the endogenous brassinosteroids suggest that the operation of the early and late C6-oxidation pathways, lead to brassinolide existence in algae. The growth and development of algae under the influence of brassinosteroids are unusually dynamic, despite the application of micromolar concentrations. These compounds regulate every aspect of algal life, from formation during development *via* stimulation of metabolite synthesis to abiotic stress responses, such as heavy metal action, salt and thermal stress. The relationship between brassinosteroids and the other well-known plant hormones has been explored. This chapter summarizes the studies of brassinosteroids on algal cultures in the last three decades.

Keywords Activity · Anti-stress Protection · Biosynthesis · Distribution

1 Introduction

Algae are autotrophic, aquatic, rarely terrestrial plants which bodies range from unicellular to multicellular structures with no vasculature and little diversification into various tissue systems. They can be a single cell as small as 1 μm (e.g. *Micromonas* sp.) or a large seaweed which can grow up to more than 65 m in length (e.g. *Macrocystis pyrifera*). Algae can produce extracellular complexing agents including polysaccharides, proteins, peptides and small organic acids that are able to decrease the concentration of bioavailable metals in the immediate vicinity of the cell. Aquatic algae are found in both fresh and salt water with a wide tolerance for pH, temperature, oxygen, and CO_2 levels. Microalgae can be used to phytoremediation techniques due to their effective efflux mechanisms for metals and the ability to modify the chemical speciation of the metal through the expulsion of inert trace

A. Bajguz (✉)
Department of Plant Biochemistry and Toxicology, Institute of Biology, Faculty of Biology and Chemistry, University of Bialystok, Bialystok, Poland
e-mail: abajguz@uwb.edu.pl

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metal complexes (Safi et al. 2014; Sahoo and Seckbach 2015; Borowitzka et al. 2016).

Algae are an area of interest due to their usefulness as food for pigments, protein, dietary fiber, mineral, vitamins, lipids, antioxidants, other valuable products and as a potential feedstock for biofuels. Microalgae can be found in the market as food supplements, colourants and food emulsions. These products come in different forms such as capsules, tablets, extracts and powder. The algal biomass is used as a supplement to noodles, breads, biscuits, candies, ice cream, bean curd and other common foods to enhance their nutritional and health values, whereas the extracts are widely used to enrich liquid foods, such as health drink, soft drink, tea, beer or spirits. Some of algal products are currently commercialized by the pharmaceutical and cosmetic industries. Nevertheless, algae are considered as nutraceuticals instead of food products due to the lack of clear and official legislations in terms of quality and requirements regarding microalgae. Algae are also a good model for laboratory studies because they grow much faster than other plants (Liang et al. 2004; Fradique et al. 2010; Sivakumar et al. 2012; Zeraatkar et al. 2016; Singh et al. 2017; Wells et al. 2017).

Plant hormones play an important role in vascular plants, coordinating growth and stress responses and regulating most of physiological and biochemical processes. Recent studies have identified genes and enzymes involved in their biosynthesis and signalling pathways. Phytohormones, including auxins, cytokinins, gibberellins, ethylene, abscisic acid (ABA), polyamines, brassinosteroids (BRs), jasmonides, salicylates and signal peptides, have been found in a variety of algae (Bajguz and Tretyn 2003; Tsavkelova et al. 2006; Tarakhovskaya et al. 2007; Bajguz 2009b; Davies 2010; Stirk et al. 2013a, b, 2003; Stirk and Staden 2014; Tran and Pal 2014; Lu and Xu 2015). Here, the recent progress in BRs detection, biosynthesis and their application for improvement of growth and resistance to abiotic stresses in algal cultures has been described.

2 Occurrence

In 1968, the first scientific account of the novel phytohormones *viz.* BRs from the leaves of *Distylium racemosum* was reported (Marumo et al. 1968). Two years later, the first bioactive compound was identified from *Brassica napus* and was named as brassin (Mitchell et al. 1970). The breakthrough discovery of the future brassinosteroid's group was the isolation of brassinolide (BL) in 1979 from the pollen of *Brassica napus* (Grove et al. 1979). Castasterone (CS), as the second BR, was isolated from the insect galls of chestnut (*Castanea crenata*) (Yokota et al. 1982). Since then, more than 60 natural BRs have been isolated from various plant species. They have been reported in higher plant species that include gymnosperms, monocots and dicots. Similarly, they have also been found in some lower aquatic (algae)

and terrestrial (bryophytes and pteridophytes) plants (Bajguz and Tretyn 2003; Bajguz 2009b; Stirk et al. 2013a, b; Stirk and Staden 2014).

Although little is known about the physiological role of BRs in algae, bioactive compounds have been detected (Table 2.1). In 1987, 24-epiCS has been identified in *Hydrodictyon reticulatum* for the first time not only in algae but also in plant kingdom (Yokota et al. 1987). To date, the presence of BL and CS was detected in 25 algal species. In many algae, e.g. *Chlorella minutissima* and *Monoraphidium contortum*, BL was present in higher concentrations than CS (Stirk et al. 2013a). Seven BRs, such as BL, CS, teasterone (TE), typhasterol (TY), 6-deoxoTE, 6-deoxoTY and 6-deoxoCS occur in *Chlorella vulgaris*. These compounds are intermediates in the early and late C6-oxidation biosynthetic pathways of C_{28} BRs (Bajguz 2009b).

3 Detection

The detection of BRs was accomplished by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) techniques. Typically, single quadrupole analysis and selected ion monitoring were used, although gas chromatography tandem mass spectrometry (GC-MS/MS) method was becoming more prominent. Nevertheless, today, liquid chromatography (LC) coupled with tandem mass spectrometry (MS) has become a powerful tool for BR analysis. The most frequently used with LC methods are triple quadrupole or time-of-flight analyzers. It is due to its selectivity and sensitivity, substantial reduction of sample-treatment steps compared to the methods above, and its reliable quantification and confirmation at the low concentrations (Kanwar et al. 2017). Using ultra-high performance liquid chromatographic separation, BRs are detected in the highly selective multiple reaction monitoring mode. The detection limit for most of the BRs analyzed was close to 50 ng/g algal biomass (Tarkowská and Strnad 2017).

Therefore, BRs are present in very low amounts in algae and both extraction and purification are important steps in detection of these compounds. BRs as neutral compounds that display no ionic properties and a high hydrophobicity are most of- ten extracted in organic solvents, such as methanol (MeOH) or acetonitrile (ACN) (Tarkowská and Strnad 2017). Briefly, after homogenization (using liquid nitrogen and ball mill) algal material is first extracted with MeOH or ACN overnight. Then, the exctract is purified using a Discovery® DPA-6S cartridges (50 mg) and Isolute® C4 SPE cartridge (100 mg). After purification, plant extract is dried in the vacuum and reconstituted in 100% MeOH. The screening process is performed on MS equipped with an electrospray ionization source coupled with LC (Tarkowská et al. 2016).

Table 2.1 Occurrence of brassinosteroids in algae

Species[a]	Brassinosteroid[b]	References
Acutodesmus acuminatus	BL (125), CS (105)	Stirk et al. (2013a)
Acutodesmus incrassatulus	BL (125), CS (93)	
Chlamydomonas reinhardtii	BL (163), CS (154)	
*Chlorella minutissima***	BL (307), CS (215), CT (41), 6-deoxo-epiCS (1580)	Stirk et al. (2013a, 2014a)
Chlorella pyrenoidosa	BL (253), CS (158)	Stirk et al. (2013a)
*Chlorella vulgaris***	BL (70), CS (470), 6-deoxo CS (320), TY (390), TE (260), 6-deoxoTY (180), 6-deoxoTE (220)	Bajguz (2009a, b)
Chlorococcum ellipsoideum	BL (169), CS (106)	Stirk et al. (2013a)
Coccomyxa sp.	BL (206), CS (177)	
Coelastrum microporum	BL (199), CS (158)	
Desmodesmus armatus	BL (125), CS (109)	
*Ecklonia maxima**	BL (stipe: 12; frond: 5), CS (stipe: 13; frond: 9)	Stirk et al. (2014b)
Hydrodictyon reticulatum	24-epiCS (0.3), 28-homoCS (4)	Yokota et al. (1987)
Gyoerffyana humicola	BL (271), CS (201)	Stirk et al. (2013a)
Klebsormidium flaccidum	BL (549), CS (429)	
Monoraphidium contortum	BL (285), CS (195)	
Myrmecia bisecta	BL (202), CS (164)	
Nautococcus mamillatus	BL (116), CS (100)	
Poloidion didymos	BL (167), CS (173)	
Protococcus viridis	BL (211), CS (135)	
Protosiphon botryoides	BL (101), CS (74)	
Raphidocelis subcapitata	BL (59), CS (59)	
Scotiellopsis terrestris	BL (337), CS (236)	
Spongiochloris excentrica	BL (131), CS (108)	
Stichococcus bacillaris	BL (292), CS (243)	
Stigeoclonium nanum	BL (169), CS (145)	
Ulothrix sp.	BL (85), CS (74)	

[a] Time of algal cultivation is 1 day, except for algae with: *2 days, **4 days

[b] Amount (:> ng/g biomass, in brackets)

4 Biosynthesis

BRs, as triterpenes (C_{30}), are generated by the joining of two farnesyl (C_{15}) chains, derived from three five-carbon isopentane (isoprene) units. The isoprenoid precursor, i.e. isopentenyl diphosphate is synthesized either from acetyl-CoA *via* mevalonic acid (mevalonate pathway) or by pyruvate and glyceraldehyde 3-phosphate (non-mevalonate pathway; present in algae). Isoprene units condensed to squalene undergo conversion *via* some steps to campesterol (Lichtenthaler 1999; Buchanan et al. 2005). Because BL and CS have the methyl group at C-24*S* position, they are synthesized from campesterol in several steps. The presence of two parallel pathways of C_{28} BR from campesterol to castasterone, named as the early and late C-6 oxidation pathways, was revealed in *Chlorella vulgaris* (Fig. 2.1) (Bajguz 2009b). These reactions are similar to pathways which exist in higher plants (Zhao and Li 2012; Chung and Choe 2013; Youn et al. 2018). Furthermore, study by Bajguz and Asami (2004) demostrates that brassinazole (Brz), specific BR biosynthesis inhibitor, inhibits the algal growth, however, the inbition effect was reversed by exogenous BL. It is known that Brz blocks the conversion of campestanol to 6-deoxocathasterone, 6-deoxocathasterone to 6-deoxoteasterone, 6-oxocampestanol to cathasterone, and cathasterone to teasterone. It suggests that the presence of endogenous BRs in algae is indispensable for their normal growth.

In *Chlorella minutissima*, 6-deoxo-epicastasterone and cathasterone occur; their initial endogenous levels increase irrespective of the presence or absence of light between 10 and 15 h of cultivation. After 15 h, a decline in BR content was observed. It suggests that light is not a controlling factor in BR biosynthesis. Moreover, a slight decrease of BR level on dark-grown *Chlorella minitussima* was observed with little increase in biomass (Stirk et al. 2014a).

5 Regulation of Growth and Metabolite Synthesis

The chemical structure of BRs is the factor differentiating the algal response on their growth and level of primary metabolites. BRs with 7-oxalactone B-ring, such as BL, 24-epiBL and 28-homoBL, are more effective than 6-ketone compounds, such as CS, 24-epiCS and 28-homoCS. BRs stimulate algal cell divisions intensively leading to an increase in the number of *Chlorella vulgaris* cells. They increase by two to three times the efficiency of the developmental cycle of *Chlorella vulgaris* and increase net photosynthetic rate and chlorophylls, carotenoids, sugar, protein, organic and inorganic phosphorus contents. BRs increase not only the content of primary metabolites in algal cells but also the intensity of sugar and glycolate extracellular secretion (Bajguz and Czerpak 1996, 1998; Bajguz 2000b). 24-epiBL has a meaningful impact on the increase of chlorophyll α and β and carotenoids such as α-, β-carotene, cryptoxanthin, lutein, zeaxanthin, astaxanthin, neoxanthin, violaxantin, content in *Acutodesmus obliquus*. 24-epiBL also inhibits the formation of

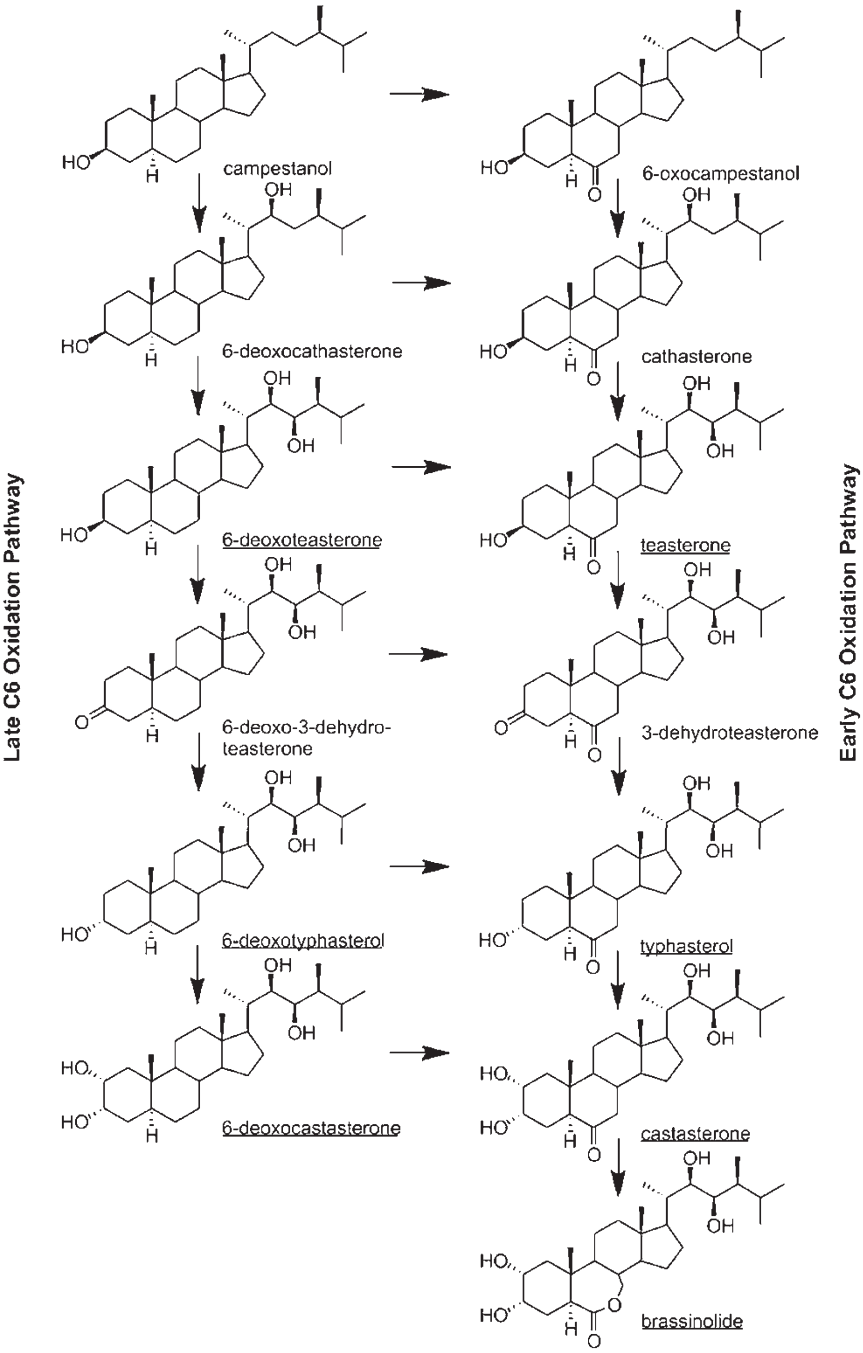

Fig. 2.1 Biosynthetic pathways of brassinosteroids (compounds detected in *Chlorella vulgaris* are underlined) (Bajguz 2009b)

reactive oxygen species such as hydrogen peroxide and oxidative damage as evidenced by a decrease of the lipid peroxidation (expressed as malondialdehyde level). The positive effect of 24-epiBL resulting from the cellular oxidative state can be alleviated by antioxidants such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and ascorbate which levels were increased by exogenous BR (Talarek-Karwel et al. 2018). BL and 24-epiBL, stimulate an increase in *Scenedesmus quadricauda* cell size. The effect was observed only at 5 nM for BL, but was seen at most of the tested concentrations for 24-epiBL. At 50 nM and higher for BL and at 100 nM for 24-epiBL reduction of cell size was observed. Both BRs increase biomass production of *Scenedesmus quadricauda* and the content of chlorophyll and carotenoids. BRs stimulate fatty acids accumulation in *Scenedesmus quadricauda*. The fatty acids profile was dependent on the type of BR and their concentration. Increasing concentrations of 24-epiBL significantly induce production of palmitic, oleic and γ-linolenic acids. Only in 5 nM, BL induces the accumulation of oleic, palmitic and palmitoleic acids. These results suggest that BRs are also important phytohormone which could be used to manipulate the fatty acids profile in the biofuel and pharmaceutical industries (Kozlova et al. 2017). Brassinazole (Brz), an inhibitor of BR biosynthesis, suppresses the growth of *Chlorella vulgaris* with a decrease in RNA, protein, sugar and carotenoids contents. The inhibitory effect of Brz was partially reversed with the co-application of BL (Bajguz and Asami 2004).

The relationship between BRs and the other phytohormones has been studied not only in vascular plants (Hardtke et al. 2007; Choudhary et al. 2012; Gallego-Bartolome et al. 2012; Hofmann 2015; Tian et al. 2018) but also in microalgae. BR induces the synthesis of ABA in *Chlorella vulgaris* cells (Bajguz 2009a). Exogenous indole-3-acetic acid (IAA) and *trans*-zeatin (*t*Z) stimulate the endogenous content of BRs in *Chlorella vulgaris* (Table 2.2). It suggests a possibility that auxin and cytokinin regulate directly the biosynthesis of BRs. Auxin and cytokinin also cooperate synergistically with BRs stimulating cell proliferation and endogenous level of protein, chlorophylls and monosaccharides in a dose-effect relationship in *Chlorella vulgaris* cells (Bajguz and Piotrowska-Niczyporuk 2013, 2014).

Table 2.2 Enhancement of brassinosteroids level by auxin and cytokinin in *Chlorella vulgaris* after 48 h of cultivation

Brassinosteroid content (fg/cell)			
	Control	50 mM IAA[a]	10 nM *tZ*[b]
6-Deoxoteasterone	0.151	0.175	0.196
6-Deoxotyphasterol	0.129	0.135	0.134
6-Deoxocastasterone	0.223	0.241	0.173
Teasterone	0.191	0.213	0.294
Typhasterol	0.251	0.267	0.245
Castasterone	0.329	0.339	0.319
Brassinolide	0.085	0.098	0.447

[a] Bajguz and Piotrowska-Niczyporuk (2013)
[b] Bajguz and Piotrowska-Niczyporuk (2014)

Application of 24-epiBL enhances the stress tolerance (e.g. temperature, light, salt stress) by increasing the level of astaxanthin in *Haematococcus pluvialis*. The eight carotenogenic genes (*ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O) were up-regulated by using different concentration of 24-epiBL. In the concentration of 25 mg/L 24-epiBL had a greater influence on the transcriptional expression of *ipi*-1, *ipi*-2, *crt*R-B, *lyc* and *crt*O than on *psy*, *pds*, *bkt*. In turn, at 50 mg/L 24-epiBL had a greater effect on the transcriptional expression of *ipi*-2, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O than on *ipi*-1 and *psy*. Furthermore, in culture treated with 24-epiBL the biosynthesis of astaxanthin (Fig. 2.2) was up-regulated by *ipi*-1 and *psy* at the post-transcriptional level, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O at the transcriptional level and *ipi*-2 at both levels. BRs, jasmonic acid (JA) and salicylic acid (SA), as anti-stress hormones, can enhance the level of astaxanthin but they have different regulatory profiles (Table 2.3) (Gao et al. 2013). Astaxanthin is used as a source of pigmentation for fish (salmons and trouts), shrimps, lobsters and crayfishes in aquaculture and for eggs in the poultry industry. Moreover, it has a higher antioxidant activity than other carotenoids. Application of this carotenoid has health benefits, such as strong antioxidant, anti-inflammatory, anti-cancer and cardiovascular effects. Astaxanthin also protects the skin against UV-induced photo-oxidation (Panis and Carreon 2016; Shah et al. 2016).

The observed increases in protein, chlorophylls and carotenoids contents due to the effects of exogenously applied BRs to the growth media would be of value in algal cultivation for commercial production of feed or bioproducts. Thus, despite the recent findings on the positive influence of BRs on algal biomass production and synthesis of valuable biomolecules, there are several gaps in our understanding of the impact of phytohormones on various features of microalgal physiology. Considering the importance of rapid growth and high metabolite content in microalgal cultivation, more study to gain a better understanding of BRs is warranted (Tate et al. 2013).

6 Anti-stress Protection

Environmental stresses are the most major natural limiting factors for plant growth and development. Most stress conditions in plants cause an accumulation of reactive oxygen species (ROS), e.g. superoxide ion, hydrogen peroxide, oxygen-containing radicals. ROS detoxification involves the combined action of both antioxidant enzymes, such as SOD, APX, CAT and glutathione reductase (GR), and metabolites, such as ascorbate, glutathione and tocopherols. Furthermore, BRs have been implicated in abiotic stress responses. Enhancement of plant resistance to various stresses by BRs has been evaluated aiming at finding practical applications for BRs in aquaculture (Bajguz and Hayat 2009; Rajewska et al. 2016).

The role of BRs in alleviating the adverse effects of stresses in algae was studied. BRs, as anti-stress substances, have generated considerable practical interest for aquacultural uses. In particular, endogenous level of BRs can be informative to

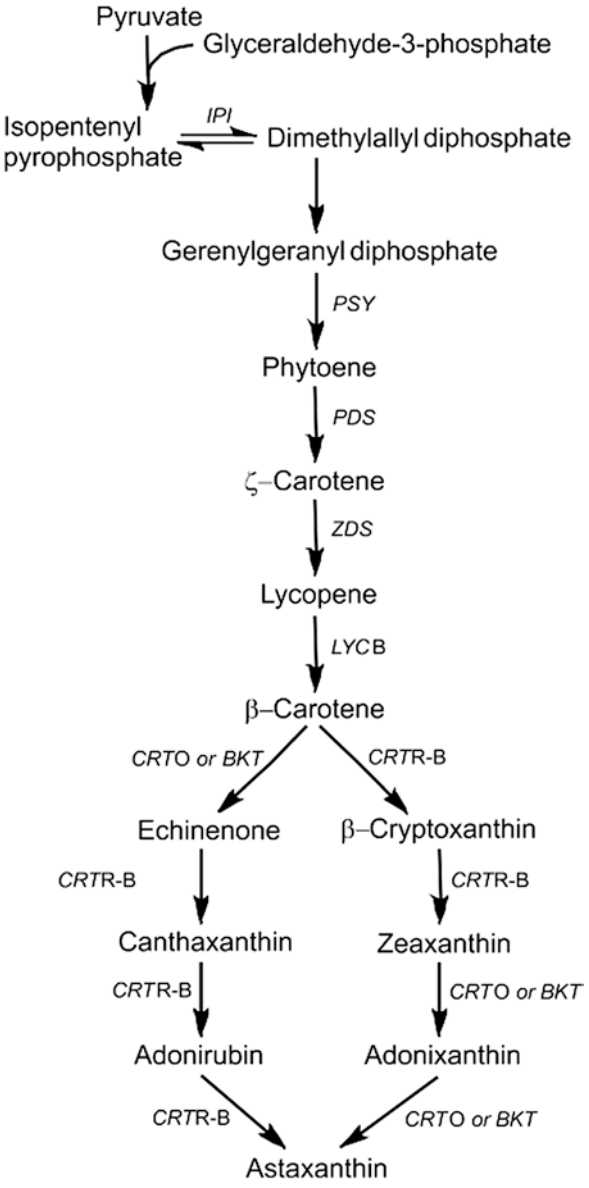

Fig. 2.2 Biosynthesis of astaxanthin in *Haematococcus pluvialis* (Gao et al. 2013). Enzyme abbreviations are as follows: *BKT* β -carotene ketolase, *CRTO* β -carotene oxygenase, *CRTR*-B β -carotene 3,3′-hydroxylase, *IPI* isopentenyl diphosphate isomerase, *LYCB* lycopene β –cyclase, *PDS* phytoene desaturase, *PSY* phytoene synthase, *ZDS* ζ -carotene desaturase

reveal key links between these hormones and stress protection as well as crosstalk with other phytohormones. Exogenously applied BL enhances the ABA content in *Chlorella vulgaris* cultures in response to short-term (3 h) heat stress (30–40 °C). BL has no significant effect on the number of cells and the content of chlorophyll and sugar in *Chlorella vulgaris* cells (Bajguz 2009a). Exogenous BL also partially overcomes the inhibitory effect of heavy metals on *Chlorella vulgaris*, decreasing

Table 2.3 Regulation of astaxanthin biosynthesis by stress-related phytohormones (Gao et al. 2013)

	Transcription level			Post-transcriptional level		
Gene	BR	JA	SA	BR	JA	SA
ipi-1	•	•	•	•	•	
ipi-2	•	•	•	•	•	
psy		•	•	•		
pds	•	•	•			•
lyc	•	•				•
*crt*R-B	•	•	•			
bkt	•	•	•			
*crt*O	•	•	•			

Gene designations are according to the corresponding enzymes, which are shown in the title of Fig. 2.2

the accumulation of heavy metals in the cells and increasing ABA, IAA and zeatin content although there was no change in the endogenous BL content (Bajguz 2011). Endogenous level of BRs increases in response to salt and low temperature (15 °C) stress in *Chlorococcum ellipsoideum*, *Gyoerffyana humicola*, *Nautococcus mamillatus*, *Acutodesmus acuminatus*, *Protococcus viridis* and *Chlorella vulgaris*. The response of algal cultures was observed within 30 min of the salt shock. The higher level of BRs, mainly CS with lower amounts of BL, 28-homoCS and TY, was shown. Furthermore, the temperature stress had a slight effect on the BRs content in these algae (Stirk et al. 2018).

The application of exogenous 24-epiBL shows increasing the content of lipids in *Chlorella vulgaris* culture under high temperature (30 °C). At the temperature of 25 °C the maximum growth rate was reached. The highest lipid content was obtained in culture treatment with 24-epiBL and growing at 30 °C. It indicates that BR significantly increases the lipid content of algae subjected to the stress induced by high temperature (Liu et al. 2018).

BL inhibits the degradation of lipids resulting from the overproduction of ROS and increase the activity of antioxidative enzymes (SOD, APX, GR, CAT) and content of antioxidants (glutathione, ascorbate) in *Chlorella vulgaris* cells treated with heavy metals (cadmium, lead, copper) (Bajguz 2010). Exogenous BRs cause the rapid response in *Chlorella vulgaris* by acceleration of phytochelatins (PC) synthesis. PC are metal-binding cysteine-rich compounds, which can facilitate the chelation of metal ions. BRs accelerate the synthesis of PC in the following order: BL > 24-epiBL > 28-homoBL > CS > 24-epiCS > 28-homoCS. Application of BRs to *Chlorella vulgaris* cultures reduces the impact of heavy metals stress on growth and enhances the chlorophyll, sugar and protein contents (Bajguz 2002, 2011). Another heavy metal detoxification mechanism is biosorption, which is dependent on pH solution. The optimum pH of metal ions sorption is between 4 and 6. Lowering the pH in cell wall spaces stimulates the growth of *Chlorella vulgaris* under the influence of BRs (Bajguz and Czerpak 1996; Bajguz 2000a). These results indicate the ameliorative influence of BRs on the inhibitory effect of heavy metals. The increase

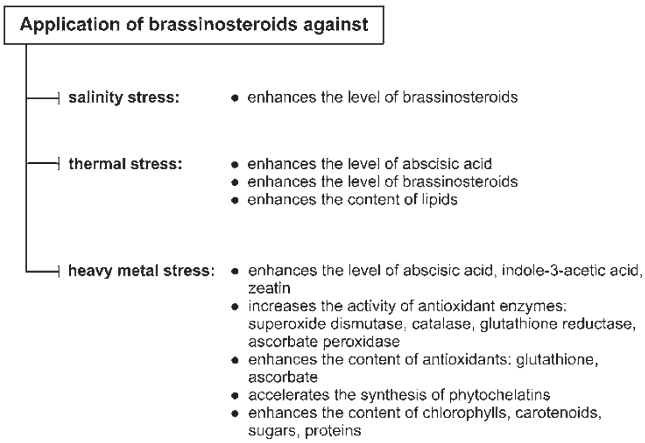

Fig. 2.3 Brassinosteroids in response to abiotic stresses in algae

of resistance due to application of BRs was reflected in the improvement of algal growth in the present of heavy metals. However, BRs are not involved by synthesising de novo in response of algal growth under heavy metal stress but can interact *via* enhancing the content of other phytohormones, i.e. auxin, cytokinin and ABA (Bajguz 2011).

Although algae have several self-defense mechanisms to survive in stressful condition, BRs regulate stress response by a complex sequence of biochemical reactions. They accelerate these processes and mitigate the negative effect of stresses in algae (Fig. 2.3).

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